

**INFLUENCE OF THE GROWTH CONDITIONS ON THE
PROPERTIES OF BACTERIAL CELLULOSE PRODUCED IN A
ROTATING-BIOREACTOR**

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Abstract

The aim of this thesis was to evaluate the impact of the growth conditions on the physio-mechanical properties of bacterial cellulose (BC) produced in a rotating biological contactor, referred here as the rotating-bioreactor. This fermentor was selected because it facilitated the manipulation of the growth conditions during BC production. BC was also produced using the alternative method known as static-culture. This method was used to establish a baseline for the strain of bacteria used. A morphological investigation of the BC produced in both static-culture and rotating-bioreactor revealed both the macroscopic and microscopic properties of the BC structure, produced by both the methods, were different. BC produced in the static-culture was made up of layers, wider cellulose ribbons and greater extent of scarring (melted BC) as compared to BC produced in the rotating-bioreactor. Their crystallinity index too was different at 89% and 83% for BC produced in static-culture and rotating-bioreactor, respectively as determined by X-ray diffraction.

The initial glucose concentration (2-100 g/L) was varied in both the static-culture and the rotating-bioreactor. Although no morphological changes were observed in the BC produced at varying initial glucose concentrations the yield of BC, was influenced by the same. It was found that the increase in glucose concentration (8-77 g/L) led to an increase in acetic acid production (19 to 255 mM). The gluconic acid production too increased from 56 to 209 mM with an increase in glucose concentration (8 to 46 g/L). The enhanced acid production impacted the yield especially at higher glucose concentrations. No BC was produced in both static-culture and rotating-bioreactor at initial glucose concentration of 100 g/L.

The growth conditions in the medium were altered by varying, the pH (3.0-6.0) at the inoculum stage in the static-culture. Due to the inadequacy of the experimental set-up,

the pH was allowed to fall naturally after adjusting the same during inoculation. There was no influence of varying pH on the morphological structure, bioprocessing efficiency (yield and rate of production) and the properties of BC produced in static-culture. Similar studies were performed using the rotating-bioreactor. Since the rotating-bioreactor had better set up for controlling the pH, the BC produced at different pH values (3.0-6.0) was controlled for the entire duration of the fermentation. It was found that increase in pH (3.0-6.0) reduced the compactness of the BC network when produced in the rotating-bioreactor. The most interesting observations were made in the BC produced in the rotating-bioreactor when the pH was not controlled. The BC was made up of macro-layers (macroscopically visible layers, also observed in wet BC) that were not observed when BC was produced at any controlled pH. The maximum yield of $0.66 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ was also obtained when the pH was not controlled. Another noteworthy observation was the change in the orientation of the micro-layers (layers observed in freeze dried and dry BC under SEM) of the BC produced in the rotating-bioreactor with a slight variation in pH from 3.0 to 4.0 and vice versa during the BC production.

The tangential velocity of the cylinders was varied by varying the RPM (2-22 RPM) to test the impact on the yield and rate of production of the BC. It was found that the rate of production increased from 1.75 to $4.0 \text{ g}_{\text{BC}}/\text{m}^2\cdot\text{day}$ with the increase in tangential velocity (0.013 to 0.16 m/s). Additionally, there was an increase in the average water holding capacity (WHC) from 92 to $176 \text{ g}_{\text{water}}/\text{g}_{\text{BC}}$ and a marginal reduction in the mechanical strength (0.03 to 0.023 MPa) of the BC produced with the increase in tangential velocity from 0.013 to 0.16 m/s .

Testing the mechanical strength of the BC in its wet form was very challenging. Different instruments and methodologies were tried without much success. Finally, a testing devise

was custom built to test the wet samples using compression under submerged conditions. The BC produced in the rotating-bioreactor without pH control had the highest mechanical strength (compared to BC produced at different pH and tangential velocity) with modulus of elasticity (MOE) ≈ 0.08 MPa. Although, this was very less in comparison to the average MOE ≈ 0.46 MPa of BC produced in the static-culture under similar conditions.

Different miscible polymers/chemicals (gelatin, chondroitin sulphate and chitosan) were tested for their ability to associate with BC when dissolved in the medium to produce a modified BC (composite). This was first tested in the static-reactor in order to determine the right concentration of the additives. A novel composite was produced using chondroitin sulphate. Its properties and bioprocessing efficiency was also determined. Additionally, the rewetting potential of both wet and dry BC produced in the rotating-bioreactor was also determined.

List of abbreviations

AFM	: Atomic force microscopy
ANOVA	: One-way analysis of variance
BC	: Bacterial cellulose
CI	: Confidence interval
CM	: Compressive modulus
CMC	: Carboxymethylcellulose
CP/MAS ¹³ C-NMR	: Cross-polarized magic angle spinning nuclear magnetic resonance
CrI	: Crystallinity index
CSLM	: Confocal scanning laser microscopy
DIW	: De-ionised water
DMA	: Dynamic mechanical analyser
ESEM	: Environmental scanning electron microscope
FTIR	: Fourier transformed infrared spectrometry
GAG	: Glycosaminoglycan
GLc-6-P	: Glucose-6-phosphate
GOD	: Glucose oxidase assay
GPC	: Gel permeation chromatography
HET -CAM	: Hen's egg test-chorioallantoic membrane
HPLC	: High performance liquid chromatography
ID	: Inner diameter
K _L a	: The coefficient of oxygen transfer
MOE	: Modulus of elasticity
MTS	: Mechanical testing system
OTR	: Rate of oxygen transfer
PEG	: Polyethylene glycol
PVA	: Polyvinyl alcohol
RPM	: Rotation per minute
SEM	: Scanning electron microscopy
TC	: Terminal complexes
TEM	: Transmission electron microscopy
UCS	: Ultimate compressive stress
UDP	: Uridine diphosphoglucose

UTS : Ultimate tensile stress

WHC : Water holding capacity

CHAPTER 1

Bacterial cellulose

1.1 Introduction

The word “cellulose” was coined in 1838 by Anselme Payne to describe the chief constituent of the cell wall in higher plants (Ottenbrite 1999). It is the most abundant polysaccharide available in nature and about 33% of all plant matter is cellulose (Sjöström 1993). It has been extensively utilized over time because of its widespread availability and unique properties. Cellulose normally occurs in trees as a composite with lignin and polysaccharides like hemicellulose, in plants such as angiosperms, gymnosperms and also in ferns and mosses (Brown 2004). It is also synthesized by both marine and fresh water algae such as *Valonia*, *Chaetomorpha* spp. In addition, certain fungi like *Saprolegnia* and *Dictyostelium discoideum* also produce cellulose (Jonas and Farah 1998). It is even produced by some marine animals like tunicates (also known as sea squirts) (Kimura and Itoh 1995). Cellulose is also produced by bacteria such as *Aerobacter*, *Gluconacetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Pseudomonas*, *Rhizobium* and *Sarcina* species (Vandamme et al. 1998). The most ancient life-forms on earth (Archaea) represented by cyanobacteria also produce cellulose (Nobles et al. 2001). The most apparent function of cellulose is protection of the cell (Brown 2004).

1.2 Structure of cellulose

The polymer structure of cellulose was first reported by Hermann Staudinger in 1920 (Staudinger 1953). Cellulose is composed of glucose monomers that are uniformly linked in β -1, 4 glucosidic bonds (a 1, 4- β -D glucan) (Fig. 1.1). These closely aligned β -1, 4-glucan chains bond with one another with the help of the available hydroxyl groups with

inter- and intra-molecular hydrogen bonds. They form aggregates of many chains also known as cellulose fibrils. Most of the cellulose fibrils derived from plants, algae and bacteria have unidirectional and laterally aligned chains of β -1, 4-glucans (Delmer and Amor 1995). The crystallographic form of such cellulose chains is termed cellulose I also known as native cellulose (made from living organism) (Ross et al. 1991). It occurs more commonly in nature and is characterised by the parallel oriented glucan chains. Cellulose also occurs in a thermodynamically stable form consisting of antiparallel glucan chains known as cellulose II, which occurs less commonly in nature. Cellulose II is not observed in plants but in a few organisms such as algae, moulds and bacteria like *Sarcinia ventriculi* (Jonas and Farah 1998). Crystallography shows that cellulose I exists in two allomorphic forms; cellulose I (α) produced by bacteria and numerous algae and cellulose I (β) derived from plants (Atalla and Vanderhart 1984; VanderHart and Atalla 1986). Cellulose also occurs in a non-crystalline form known as nematic ordered cellulose (Kondo et al. 2004).

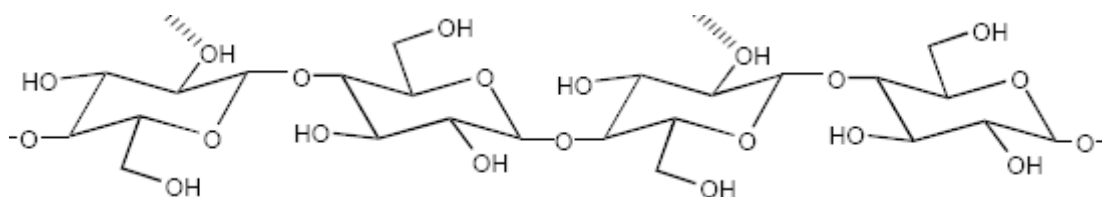


Fig. 1.1 – The basic structure of cellulose molecule. Adapted from Klemm (2005).

1.3 Bacterial cellulose (BC)

Cellulose is tasteless, odourless, hydrophilic, insoluble in water and most organic solvents and biodegradable. The cellulose microfibrils have high tensile strength, comparable to

steel due to strong hydrogen bonding (Ross et al. 1991). By virtue of these properties, cellulose imparts an exceptional strength and chemical resilience to the tissues in which it resides. Many industries, like textile and paper, have capitalized on plant-based cellulose because of these unique properties. The ever-increasing industrial demands on plant-based cellulose have put a pressure on plant biomass sources. In the present time, preservation of natural resources is gaining importance and people are constantly looking for alternatives to ease the demand on natural resources. A very good substitute for plant-based cellulose is bacterial cellulose (BC). Although it cannot replace plant-based cellulose totally, it can provide a good alternative for high-end applications that require greater levels of chemical purity. The properties of high water retention, mouldability and an apyrogenic nature make it very attractive for the medical-based industries. While a number of species of bacteria can produce BC, the one that justifies commercial production is *Gluconacetobacter xylinus*. The bacteria of this genus are obligate, Gram-negative aerobes found on food such as fruits, vegetables, in vinegar, fruit juices and alcoholic beverages (Klemm et al. 2001).

1.4 From cell to cellulose

Cellulose is one of the final products of the carbon metabolized by *G. xylinus* besides cell mass, carbon dioxide and/or acid. In the past it was hypothesized that the cellulose is formed at a distance from the bacterial cell (Colvin 1972) but it was later proved that it is formed closer to the cell (Brown et al. 1976). The synthesis of uridine diphosphoglucose (UDP-glucose) the precursor of cellulose (Swissa et al. 1980) is housed in the bacterial envelope (Cooper and Manley 1975). This envelope is also characterized by the presence of about 50-80 pores (Fig. 1.2) of approximately 10 nm in size situated on the outer lipopolysaccharide membrane (Zaar 1979). These pores were observed only along the longitudinal axis of the cell envelope and not at the tips (Brown et al. 1976). There are

two hypothesis for the proposed function of these pores, one for the extrusion of glucan chains synthesized within the bacterial cell envelope (Zaar 1979). The other is to function as sites that actually help in the crystallization of the glucan chains into sub-elementary fibrils that organize into microfibrils outside the envelope (Zaar 1979) (Fig. 1.2). There is an agreement among the groups that alongwith the pores, the cell envelope is also characterized by the presence of linearly placed arrays of terminal complexes (TC) (Brown et al. 1976; Hirai et al. 2004; Zaar 1979). These TC's are made up of cellulose synthesizing protein units that give rise to ordered parallel glucan chains and are extruded through the pores and assemble as microfibrils outside the envelope (Brown et al. 1982; Brown et al. 1976; Haigler et al. 1982; Tokoh et al. 1998; Zaar 1979). The rate of excretion of a single microfibril was calculated as 2 μm per minute (Brown et al. 1976; Hirai et al. 1997) and 2.5 μm per minute by Kai et al. (1982). Chemically these microfibrils are made up of linear polymers of D-anhydroglucopyranose or glucose units connected by β -1,4-glycosidic bonds (Jonas and Farah 1998). The lateral width of these microfibrils was estimated as 3 nm by Colvin (1963). The average area of cross-section of a solitary fibril was estimated approximately as 1.6×5.8 nm by Brown et al. (1976), 4×80 nm by Zaar (1979) and more recently 1×16 nm by Astley et al. (2001). The difference in estimation of size by each group could be due the different methods of observation. These microfibrils are further subjected to lateral intermolecular H-bonding to form aggregates known as cellulose ribbons (Fig. 1.2) and are directed parallel to the longitudinal cell axis (Benziman et al. 1980; Brown et al. 1976; Yamanaka et al. 2000). The ribbon structure proposed by Astley et al. (2001), suggests densely packed microfibrils that are made up of crystalline cellulose and are coated by polymer chains (non-crystalline). The number of microfibrils present in each ribbon and their arrangement has not been confirmed. Some studies suggest that there are 46 microfibrils

per ribbon (Brown et al. 1976), while another suggests a range between 20-50 per ribbon (Colvin 1966) a more recent study estimated it as 5-10 microfibrils per ribbon (Gelin et al. 2007).

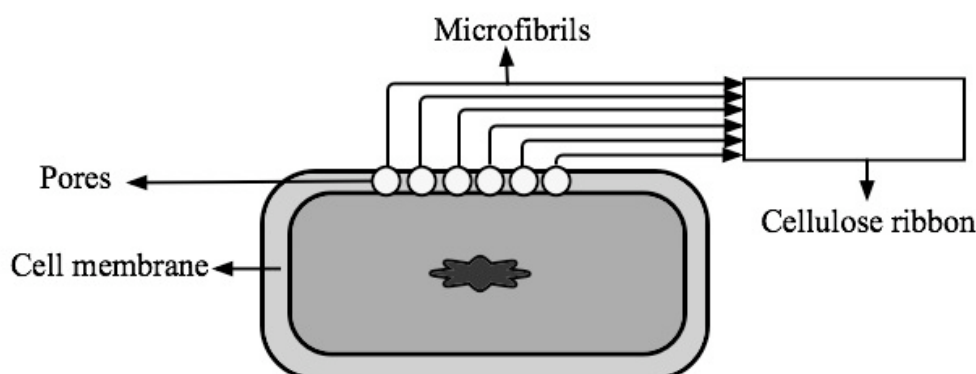


Fig. 1.2 – A schematic of *G.xylinus* extruding cellulose from the pores present in the outer cell membrane to form microfibrils and subsequently cellulose ribbon with hydrogen bonding. Adapted from Klemm (2001).

The newly formed ribbons from different bacterial cells form a part of the network called the pellicle. The various steps involved in ribbon formation adapted from Brown et al. (1976) have been summarised in Fig. 1.3. The size of the ribbons reported by Brown et al. (1982) is 3.2 x 133 nm and 4.1 x 117 nm, as reported by Yamanaka et al. (2000). The polymerisation of cellulose followed by crystallization leads to the forward propulsion of the cell by the rotating motion on its longitudinal axis when the ribbon gains sufficient length to bond with other ribbons (Brown et al. 1976). This also indicates that cellulose biosynthesis and cell movement are closely inter-linked (Brown et al. 1992). If the viscosity of the medium is changed by addition of water soluble polymers such as polyvinyl alcohol (PVA) or polyethylene glycol (PEG), the movement of the bacterial cells is affected and the formation of cellulose microfibrils gets impacted due to reduced

cell mobility (Shibazaki et al. 1998). The cellulose ribbons aggregate to form an entangled mesh termed the cellulose pellicle. The size of the pellicle is limited solely by the size the surface area of the vessel used for BC production.

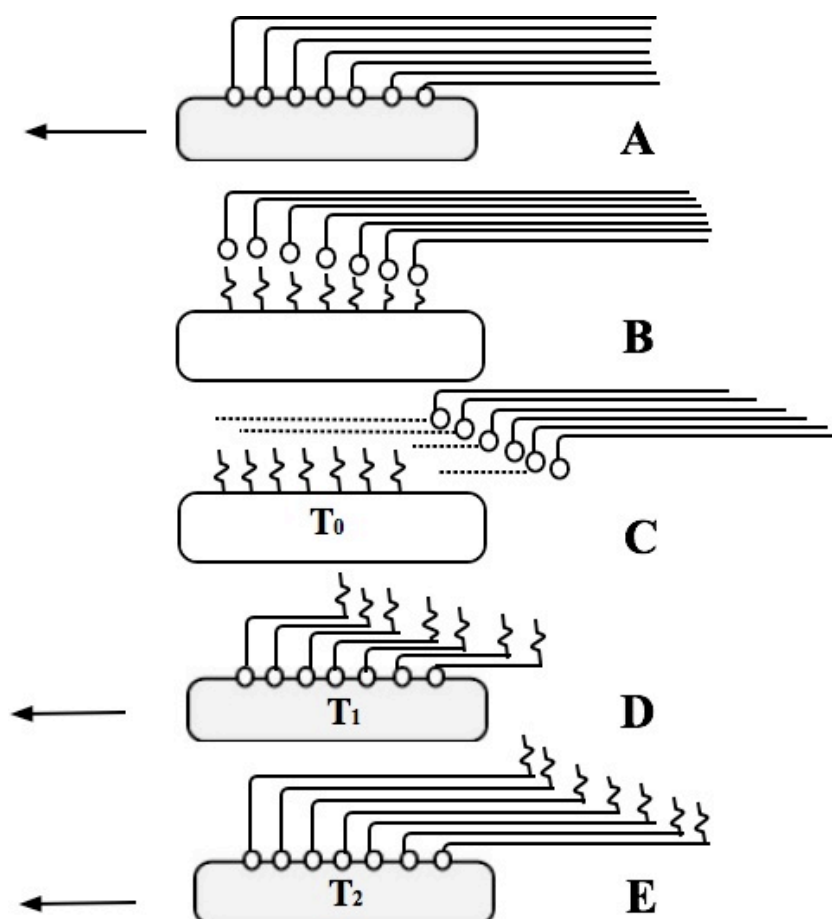


Fig. 1.3 – The schematic diagram of the series of events that occur during extrusion of cellulose ribbons, movement of the bacteria away from the pellicle and the subsequent re-growth of cellulose. The arrows represent the direction of movement of the bacteria and T represents the time and the subscript refers to the unit in minute (T_1 = one minute). A) Ribbon composed of microfibrils extruded from the bacteria is connected to the pellicle. B) The resultant movement of the bacteria tears the microfibrils at the pores forming spring like lateral projections. C) The bacteria gets separated from the ribbon that

connected it to the pellicle. D) Re-extrusion of microfibrils causing the lateral extensions to move away from the bacterial surface. E) Regrowth of cellulose ribbon along with the bacterial movement. Adapted from Brown et al. (1976).

1.5 Properties of bacterial cellulose

BC in its never-dried form is quite hydrophilic in nature and can absorb nearly 100 times the weight of cellulose, but upon air drying it becomes hydrophobic in nature and can absorb as little as 6% water. White and Brown (1989) explained this hydrophilic nature is a result of the porous structures present within the interior surface area of the never-dried BC pellicle. Drying affects this hydrophilic nature making it hydrophobic and this change is irreversible. This is attributed to the increase in the cross-linking due to the secondary bond formation that resist rupture during rewetting (Westman and Lindström 1981). The BC is known for extraordinary shape retention and can be produced in almost any shape, depending on the container used in BC production (White and Brown 1989). The porous nature of the BC helps the rapid absorption of dyes or other chemicals post-production (White and Brown 1989). The BC has exceptional mechanical properties due its crystallinity and microfibrillar structural properties; its tensile strength is approximately four times greater than any organic fibre. The density of dry BC is 1.59 kg/m³ (Sugiyama et al. 1991). Cellulose I exists in two polymorphic forms α and β and there have been no reports of pure samples of cellulose I(β) only of cellulose I(α) (O'Sullivan 1997)

Crystallographic investigation by CP/MAS ¹³C-NMR analysis on freeze-dried samples of dry BC confirm the presence of cellulose I(α) (Watanabe et al. 1998a). Reports suggests that BC has approximately 70% of cellulose I(α) as compared to cotton at 20% (O'Sullivan 1997).

1.6 Comparison of bacterial and plant based cellulose

The bacterial cellulose has the same molecular formula as the plant-based cellulose but quite different macroscopic properties. The cellulose formed by all known bacteria is extracellular in nature in contrast to the plant-based cellulose, which is an intrinsic part of the cell wall matrix (Fig's. 1.4 and 1.5). The plant cell converts carbon from the carbon dioxide released during the process of photosynthesis into cellulose. The bacteria can utilize carbon from sugars such as glucose, fructose lactose, D-galactose, mannitol and other organic sources such as ethanol and glycerol (Panesar et al. 2009;Tarr and Hibbert 1931;White and Brown 1989). The rate of cellulose biosynthesis of bacteria is approximately forty times faster than that of cotton plant (Brown 1991). The BC has a very high purity level and is not associated with substances like hemicellulose, lignin and/or pectin unlike plant based cellulose (Fig.1.4). Cellulose from wood pulp has typical chain lengths between 300 and 1700 units; while bacterial celluloses have chain lengths ranging from 800 to 10,000 units (Klemm et al. 2005). The outstanding properties of BC in comparison to the plant-based cellulose are that the BC is extremely hydrophilic in nature (never dried form) and it has a mechanical strength greater than plant-based cellulose such as paper pulp and cotton (Brown et al. 1992). Some of the other properties adapted from Iguchi et al. (1988) are summarised in Table 1.

Table 1. A comparison of properties of BC with plant-based cellulose (paper).

Properties	Bacterial cellulose	Plant cellulose (paper)
Young's modulus	13.6 GPa	2.4 GPa
Density	1060 kg /m ³	565 kg /m ³
Sonic velocity	3580 m /sec	2060 m /sec

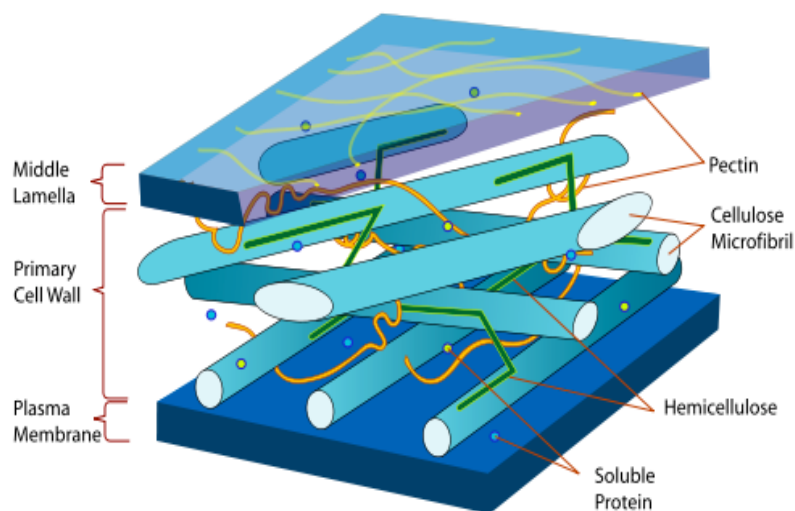
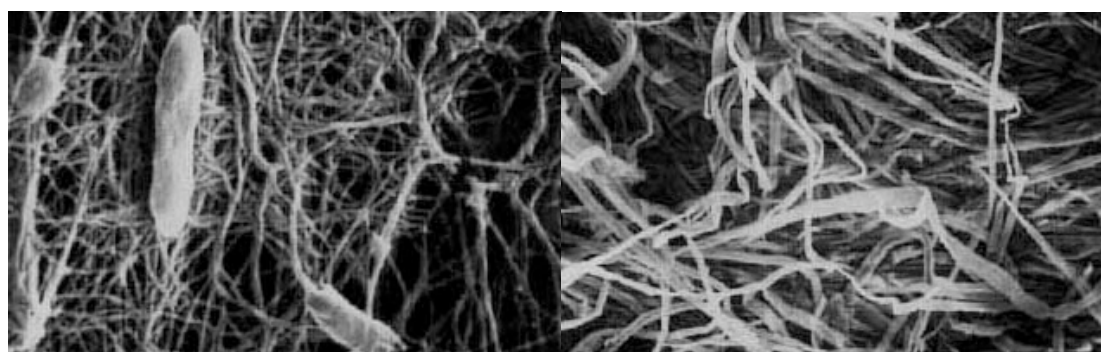


Fig. 1.4 - The arrangement and location of cellulose microfibrils in a plant cell wall (Villarreal 2008).



Bacterial cellulose ($\times 20,000$)

$2\ \mu\text{m}$

Plant cellulose ($\times 200$)

$200\ \mu\text{m}$

Fig. 1.5 – A SEM micrograph comparing BC and plant cellulose (Ano and Sugano 2001).

1.7 Products of *G.xylinus* other than BC

There have been reports that strains of *G. xylinus* not only form BC and acids such as gluconic and acetic but also an exopolysaccharide called acetan (Couso et al. 1987). The major components of this polysaccharide are glucose, mannose, glucuronic acid, and

rhamnose in a molar ratio of 4:1:1:1. In a related study, Jansson et al. (1993) used NMR to investigate the structure of acetan and found it to be similar to that reported by Couso et al. (1987). Certain variants of acetan were also reportedly produced by different strains of *G. xylinus* (Maccormick et al. 1993). In another study, it was reported that low glucose concentration reduces the production of gluconic acids while increasing the rate of acetan production (Kornmann et al. 2003).

1.8. History –discovery of BC till the more recent developments

The first ever report of cellulose formation by bacteria was published by Adrian Brown in 1886. He made this observation whilst studying the gelatinous, mat-like formation on the surface of the broth during vinegar fermentation. On analysis, these translucent structures were confirmed as cellulose. The micro-organism that made this buoyant cellulose film was named, *Bacterium xylinum* “xylinum” meaning cotton-like, (Brown 1886). Recently this bacterial species was reclassified as *Gluconacetobacter xylinus* (Yamada 2000).

After the discovery and subsequent naming, research groups all over the world started investigating the morphological and physiological properties of this newly discovered bacterium. The first ever scientific study on *G. xylinus* was published by Tarr and Hilbert (1931). This group concentrated on optimum growth and BC production of *G. xylinus* on assorted substrates including pentoses, hexoses, glycerol, galactose and mannitol. Their findings proposed that neither pentose sugars nor addition of methyl group to hexoses sustained cellulose production. This group further devised an ideal growth medium to promote cellulose production and proved that ethanol addition to the medium enhanced production of cellulose.

After another decade of silence, many research proceedings on *G. xylinus* began to appear in various journals. The group that made the maximum contribution to the initial research work was Hestrin and his co-workers from 1946-1963. They reported the physical characteristics of the BC as a meshwork of individual fibrils as seen under a microscope (Aschner and Hestrin 1946). They were also the first to report the fact that the mat-like cellulose was produced only in still, standing cultures and that agitation formed irregular round blobs with a lower production rate (Schramm and Hestrin 1954b). They demonstrated for the first time that these bacteria were aerobic in nature. They also

proposed that oxygen tension limited BC formation at the liquid-air interface in undisturbed static cultures. They also confirmed the optimal pH range for cellulose production at between 5 and 7. Analysis of the BC using infrared absorption, unequivocally identified it as a high-molecular weight, native, crystalline cellulose I (Hestrin and Schramm 1954b). They investigated biosynthesis of cellulose and reported that cellulose production and respiration occurred at the same time but through alternate pathways. They also suggested that some constituent of the outer surface of the bacteria was a probable catalyst of the production of cellulose.

In the paper published in 1957 and 1963, a detailed account of the biosynthesis pathway was established showing the different substrates and the inhibitors of cellulose production and that this final product was formed from citric-acid cycle intermediates (Gromet-Elhanan. and Hestrin 1963;Schramm et al. 1957b). While Hestrin and his group were interested in the various factors affecting cellulose production, there were other groups focussing on purely the structural aspect of both *G. xylinus* and BC. Using the newly introduced electron microscopy technique, Wyssling and Muhlethaler (1946) observed the structure of BC and reported darkened thread-like structures of similar diameter (20 nm) in greyish films of pure cellulose. A similar observation was made by Kaushal et al. (1951) except that they found that the diameter of the fibres were dissimilar and ranged between 20-25 nm.

1.9 Nata de Coco, a bacterial cellulose-based dessert

A cottage industry manufacturing a sweet dessert known as “Nata” was flourishing in Philippines oblivious of the fact that they were using the same bacteria to make a delicious dessert. Nata (derived from Latin word *natare* which means “to float”) still remains a popular dessert food produced in the Philippines from fruit juices or other

plant-based extracts. Lapuz et al. (1967) established that the Nata produced from fermenting coconut water was indeed produced by *G. xylinus*. Independent of the scientific research involving the bacteria, there were research groups investigating different means of optimising the Nata production. Some of the published work includes study of the optimal parameters for Nata production (Alaban 1962; Lapuz et al. 1967) and some genetic work involving strain selection of bacteria with higher Nata production rate (Gallardo-DeJesus et al. 1971). They screened and identified 33 different strains of cellulose-producing bacteria, and further isolated specific strains to produce different textured and higher yields of Nata.

A popular beverage consumed in Asia, Russia, and Central Europe called Kombucha tea is produced from a mixed culture of *Gluconacetobacter* and yeast species (Sievers et al. 1995). The beverage is produced with a mixed culture on black tea and sugar also known as tea fungus fermentation.

1.10 Probable reasons for bacterial cellulose production

Studies related to the structure, production and synthesis of BC have been published since 1930. Different research groups have hypothesized the reason for bacterial cellulose production but very few have demonstrated the same. Williams and Cannon (1989) suggested that BC was produced in order to create a buoyant surface supporting the cell growth at the liquid-air interface and provide protection from harmful ultraviolet (UV) rays. The BC provides an entrapment that enhances cell adhesion and the nutrient absorption capacity of the cells (Jonas and Farah 1998). While other groups suggested that BC facilitates the process of cell adhesion necessary for interaction similar to those seen in *Rhizobium* and *Agrobacterium* spp. (Ross et al. 1991). Okamoto et al. (1994) proposed that BC acted as a reserve for starving bacteria. During shortage of food, BC

could be decomposed with the help of exo- and endo- glucanases, whose presence has been detected in some *G. xylinus* cultures. Some other groups focussed on the shielding aspects of the BC against undesirable environmental changes including reduced moisture, pathogens, toxins, change in pH and the most important being protection from ultraviolet radiation. A plausible reason was demonstrated by Ross et al. (1991). They subjected bacteria entrapped in BC to ultraviolet (UV) light for 1 hour and found 23% survival and on removal of the BC, was reduced to 3%. Another group recently demonstrated that the cellulose pellicle protected the bacterial cells against damage caused due to freezing at very low temperatures (Evans and O'Neill 2005).

1.11 Biosynthesis of cellulose

Despite the efforts of many scientists, the biosynthetic pathway of cellulose in plants is not yet elucidated. The discovery of bacterial cellulose and the subsequent unravelling of its biosynthetic pathway of the cellulose production encouraged researchers to draw parallels with plant cellulose. The first such published attempt was made by Marx-Figini and Pion (1974). The plant-based cellulose is a uniform structure of regular molecular weight unaffected by the usual biosynthetic parameters such as cellulose yield, reaction time and conditions. But this is not true of the cellulose produced by bacteria as confirmed by Marx-Figini and Pion (Marx-Figini 1982;1974). In bacteria, the synthesis of cellulose is a complex, multiple step process involving a number of enzymes and various proteins that are both catalytic and regulatory in nature. It is closely associated with the catabolic process, oxidation and utilizes about 10% of the energy derived from catabolic reactions (Weinhouse 1977). It does not inhibit any of the anabolic processes, including protein synthesis (Ross et al. 1991). According to published reports, up to 200,000 glucose molecules per second are synthesized into β -1, 4-glucan chains per *G. xylinus* cell (Hestrin and Schramm 1954b).

In *G. xylinus*, cellulose is the ultimate product of the metabolism of carbon from either the pentose phosphate cycle (depending on the physiological status of the cell) or together with gluconeogenesis (Ross et al. 1991; Tonouchi et al. 1996). It does not synthesize the enzyme phosphofructose kinase essential for glycolysis, hence it cannot metabolize glucose anaerobically (Ross et al. 1991). *G. xylinus* converts a number of different carbon compounds such as hexoses, glycerol, dihydroxy-acetone, pyruvate and dicarboxylic acid into cellulose with about 59% efficiency. The BC production depends on simultaneous oxidation processes and not on protein synthesis. This is evident from cells producing BC in the presence of carbon substrate even when deprived of nitrogen source (Weinhouse and Benziman 1972).

There were various published studies aimed at unravelling the biosynthetic pathway of BC production. Colvin and Leppard (1977) studied *G. xylinus* and *A. acetigenus* and suggested a biosynthetic, cyclic pathway in which the glucose gets converted into glucose-6-phosphate, which then converts into glucose-1-phosphate and then uridine diphosphoglucose (UDP), and finally into cellulose (Fig. 1.6). This pathway can be supported by the comparative studies of Swissa et al. (1980) using radio-labelled carbon in non-cellulose producing mutant and the wild type bacterial cells.

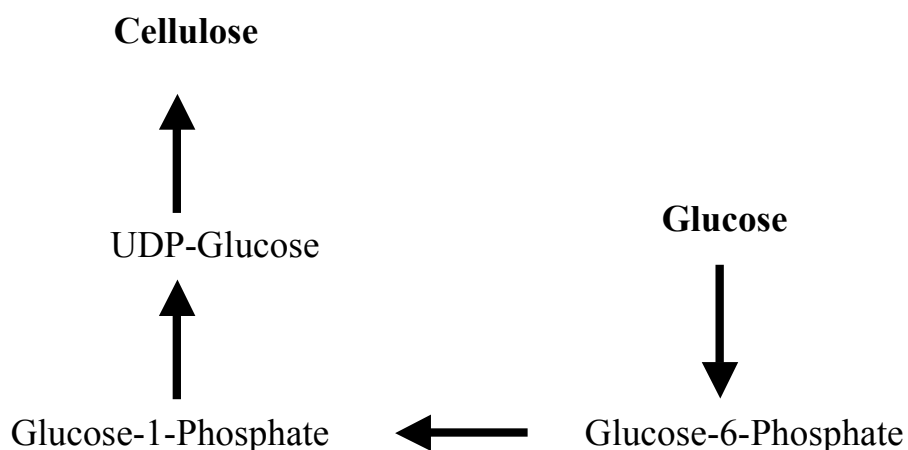


Fig. 1.6- A simplified pathway of BC cellulose synthesis from glucose. Adapted from Serafica (1997).

The conversion of glucose into cellulose by *G. xylinus* can be summarised by the following four enzymatic steps (Hestrin and Schramm 1954b; Ross et al. 1991).

- Phosphorylation of glucose by glucokinase.
- Isomerisation of glucose -6-phosphate (Glc-6-P) to glucose-1-phosphate (Glc-1-P) by phosphoglucomutase.
- Synthesis of UDP-glucose (UDPG) by UDPG-pyrophosphorylase.
- Cellulose synthase reaction.

The precursor of cellulose is UDP-glucose, the product of the traditional pathway that is similar to many organisms and plants. It then involves glucose phosphorylation into glucose-6-phosphate (GLc-6-P) that is further catalyzed by glucokinase. This newly formed intermediate is isomerised to Glc- α -1-P and then catalyzed by phosphoglucomutase. The metabolite produced is converted into UDP-glucose by UDP-glucose-pyrophosphorylase. The pyrophosphorylase enzyme is imperative for BC formation and is the basis of distinguishing between BC producing cells and the non-producing mutants (Valla and Kjosbakken 1982). These mutants show cellulose synthase

activity although they do not produce BC due to the deficiency of pyrophosphorylase, this was proved by *in vitro* assays conducted by Saxena and Brown (1989). Additionally the activity of this crucial enzyme varies within different strains of *G. xylinus* and is the basis for determining the strain specific efficiency of BC production. The UDP-glucose is converted into cellulose via a direct substitution mechanism during cellulose synthase reaction. This conclusion was based on the failure to identify any intermediates during an *in vivo* labelling assay conducted with [C14] glucose (Delmer et al. 1983).

1.12 Different methods of bacterial production

The BC production can be broadly classified into three different methods viz. static bacterial culture method (static-culture) in which the BC is produced at the air medium interface under static or stationary conditions. The rotating biological contactors (rotating-bioreactor) in which the BC is produced on partially submerged plates or cylinders in the medium and the agitated bacterial cultures method (agitated-bioreactor) where the BC is produced as disintegrated flocs within the medium of a traditional fermentor. Different groups have used one of the above methods or a combination to develop bioreactors for BC production.

1.12.1 Static bacterial culture method (static-culture)

This is a very simple method in which medium with a carbon substrate is inoculated and left undisturbed in a vessel. The cellulose forms as a pellicle floating at the air/water interface and is harvested when the desired thickness of BC is reached. This is the well known method for commercial production of Nata de Coco in the Philippines.

1.12.2 Production of Nata

The BC production for Nata de coco is quite simple. The fermentation medium typically consists of carbon source usually as cane sugar, fresh coconut milk, glacial acetic acid,

water and inoculums (previously cultured on the medium). The process of mixing the inoculums and the medium is done in non-aseptic conditions in plastic drums and then poured into polycarbonate trays. The trays are stacked with cardboard sheets covering each tray and stored in incubation rooms that are temperature controlled with light bulbs. A desired amount of medium is taken and used as an inoculum for the succeeding fermentations. The trays are left undisturbed for 10-14 days till the desired thickness of the pellicle is reached and then harvested and washed in clean water by soaking. This cleaned BC pellicles are cut into the desired shapes and boiled in water to kill the bacteria. After cooling and draining, sweeteners such as sugar and colouring and/or artificial flavours are added.

There was no evidence as to when it was introduced in the Philippines but there were claims that it was about a century old (Africa 1949). In search of a more suitable medium for the Nata organism (*G. xylinus*), Africa proposed using waste coconut water from copra processing while working in the Microbiology Laboratory of the National Coconut Corporation. Proper adjustment of the sugar concentration and the pH of the medium greatly improved the Nata formation making the process feasible. Much work was done by the National Coconut Corporation on the optimization of Nata production including, sugar concentration and the pH of the media. Their work gave a boost to the Nata production and industry at large (Lapuz et al. 1967).

1.13 The Ajinomoto process

Another variation of the static culture method is reported by the Japanese company, Ajinomoto (Okiyama et al. 1992b). The process involves two-stage fermentation for producing BC in pellicle form. The first stage involves an air-lift bioreactor to generate a

high cell density inoculum that increases rate of production. This broth is then transferred to trays similar to those used in Nata production.

1.13.1 The modified static-culture method

Sakairi et al. (1998) attempted to scale up the static culture production. They introduced two shallow pans made of stainless steel in the larger culture trays. These were connected to winding rollers. The BC was produced in either of the two pans and after two days of incubation passed through the winding roller and bath of 2% sodium dodecyl sulphate (to denature the bacterial cell wall). Fresh medium was added every 8-12 hours and the fermentation was extended for multiple weeks. This apparatus was placed in a temperature controlled incubator with filtered air. The BC produced by this method was shown to have comparable properties (filament structure and mechanical strength) to that produced in traditional static cultures.

1.13.2 Advantages and disadvantages of the static-culture method

Although static-culture appears attractive due to its simplicity, it is not a feasible option for large-scale production due to the high cost of labour involved (Schrecker 2005). Additionally, it is not a very good tool for investigative studies on BC production in a laboratory environment. This is because the static cultures have to be left undisturbed during pellicle formation. And even after the pellicle has formed, it is a challenge to even measure the pH of the medium let alone control the same. Thus, controlling important growth parameters such as pH and substrate concentration is challenging in this method. It has been postulated by Borzani and Desouza (1995) that the BC pellicle increases in thickness due to the active cells on the uppermost level. Thus after a point, the cells lack access to medium due to the decreasing substrate flux through the increasing thickness of the pellicle under formation.

1.14 The agitated bacterial culture method (agitated-bioreactor)

In this method, the BC is produced in a totally submerged, well-mixed medium. This method was first reported by Schramm and Hestrin (1954a). They produced BC in shake flasks to increase aeration throughout the volume and thus produced BC under agitated conditions. They found that under agitated conditions, *G. xylinus* did not form a pellicle, but small irregular sized spherical balls or flocs. Its potential to be scaled up inspired further research. The rate of BC production in such agitated cultures was two fold higher than the traditional static culture method (Setyawati et al. 2007). Using the same principle, Chao et al. (2000) developed a 50 L internal-loop airlift reactor that claimed to give increase productivity at reduced power consumption under optimum conditions.

There were issues with such agitated fermentor that included inadequate mixing of the medium due to high viscosity. In addition, higher oxygen transfer rates required higher agitation rates that resulted in sheer stress on the BC produced. Some reactor-based drawbacks such as BC fouling at the impeller was reported by White and Brown (1989), but the major problem seemed to be the mutation of the BC producing cells into non-producing mutants.

1.14.1 Modified agitated-bioreactor (PCS biofilm reactor)

In a recent study, Cheng et al. (2009) modified the agitated-bioreactor by attaching 12 hollow tubes (O.D=10.5 mm thickness=2.5 mm) made of plastic composite (PCS) to the agitator shaft in a woven mat-like fashion with six rows of two parallel tubes. The BC produced outside these tubes showed an increase in rate of production by 2.5 times and enhanced properties such as, mechanical strength and crystallinity as compared to the pellets produced in the regular agitated-bioreactors.

1.15 The rotating biological contactor (rotating-bioreactor)

Rotating biological disc reactors are used for the treatment of domestic or industrial wastewater, first reportedly used in Germany, 1920. The first reported study of BC production using a modified rotating biological disc reactor was published by Sattler and Fiedler (1990). They used a rotating-cylinder made up of roughened glass in the medium to produce BC on the cylinder surface (air-medium interface). This fermentor was named “Walzenfermentor”. They reported a two-fold increase in the cellulose production as compared to the static-culture. There are more recent reports where rotating-bioreactor with cylinders were used for various BC production related studies (Kim et al. 2007; Krystynowicz et al. 2002; Mormino 2001). These studies used cylinders in order to get sheet-like pellicles that could be cut into desired shapes.

The rotating cylinder is replaced with discs in a study by Serafica (1997). A desired number of discs, connected to a shaft, are rotated through a medium inoculated with the *G. xylinus* inoculum. The surface of the disc is roughened to provide for easy attachment of the cells and the medium level is ideally maintained at fifty percent. The cells then begin to grow on the disks and get nutrients and air during subsequent rotation through the liquid and air phase and begin the production of BC on the surface of the discs. The rate of BC production is directly proportional to the area of the air/liquid interface (Masaoka et al. 1993). A rotating-bioreactor provides more surface area (5 to 100 times) compared to the static culture method in the same volume of medium (Serafica 1997). Additionally, the rate of BC production per unit area can be increased by adding more discs. This high surface area per unit volume can enable large scale-production of BC. The other advantages of this method include improved process control of essential environmental parameters for optimum BC production, including, pH, sugar concentration, and temperature and/or pressure control. Although this is possible in

agitated culture method too, the final product in this case is discs that can be cut to the desired shape and size, unlike the agitated culture method where the product is irregular or spherical shaped flocs. This design also permits the addition of co-polymers, dyes or any such additives for the production of novel composites of BC (Serafica et al. 2002).

1.16 Other novel reactors

Gostomski et al. (2002) proposed a dripping film reactor to improve volumetric productivity. The growth medium was trickled on to a hanging sheet but it suffered from poor medium distribution. Another novel approach was made by Onodera et al. (2002) using silicone bags (wall thickness: 0.15 mm) submerged in growth medium. BC was produced on the outside of the oxygen permeable bags while air was continuously passed through the bags. This was one method in which virtually the entire medium could be utilized for BC production. Thus, the surface to volume ratio could reduce production costs when compared to static cultures method. Another advantage of this method was that the BC could be moulded into any desired shape. This method has been utilized by Klemm et al. (2001) to produce micro vessels of different diameters.

1.17 Aerosol reactors

In yet another attempt to modify the static culture method, Hornung et al. (2007) developed two different types of aerosol reactors. In the substrate aerosol reactor, BC was produced in two removable sterilized containers filled with the growth medium. These level controlled containers were filled with medium by gravity from the feed tank. Medium and air was sprayed periodically (flow rate=10 ml/min) in the form of spray by the vibrations from an ultrasound device. The spraying was done on the upper surface of the reactor, where the active cells produced BC. The substrate aerosol reactor was further modified to ensure even distribution of the medium throughout the growth surface of the

medium. This was achieved by introducing a roof-shaped distribution box placed above the culture box. The medium was fed through an eight-channel distributor that sprayed medium periodically onto the distribution box. These aerosol reactors ran for longer duration of time (six weeks) compared to the traditional reactors. The direct supply of substrate to the active cells overcame the diffusion problem in static-culture method, however these aerosol reactors suffered from severe contamination issues.

1.18 Factors affecting the BC production

BC production rate depends on surface area and remains unaffected by volume and depth of the fermentation vessel for the static-cultures and the rotating-bioreactor systems with the exception of agitated-bioreactors (Masaoka et al. 1993). Other factors affecting BC production are broadly divided into four categories: the strain of the bacteria, the growth medium, the different methods of production and operational parameters (pH, dissolved oxygen, temperature, and concentration of sugar). The strain is very important because some strains are genetically predisposed to produce BC at a faster rate, while some are genetically altered to induce the same effect. The BC production rate also depends on the choice of carbon and nitrogen substrates and the addition of trace elements to the medium. The method of production and/or reactor design is another important aspect in the rate of BC production because some methods such as agitated give a higher production rate per unit volume compared to the static culture method. The operational parameters of the method selected are also important because not only do they affect the rate of production but could also the physical attributes of the BC (Kato et al. 2007). The optimum parameters depend on method and/or reactor design used and addition of chemicals.

1.18.1 Strain of bacteria

The strain of *G. xylinus* is very important for BC production because some strains are overproducers, while some are non-producers, while others can be genetically altered to do the same. Masaoka et al. (1993) investigated various strains of *G. xylinus* and the genus *Agrobacterium* to find the strains that produce maximum BC. Only four strains of *G. xylinus* were found promising of the forty one strains studied. There are reports of discovery and/or development of overproducing bacteria. *A. xylinum* KU-1, was able to produce BC from D-mannitol at rate that was three times higher than from glucose under the same conditions (Oikawa et al. 1995). DeWulf et al., (1996) genetically engineered a strain of *G. xylinus* that produced less gluconic acid compared to the wild-type strain. This enabled the cells to produce less acid during BC production and maintain better pH for improved productivity. The pellicles produced were double in size to that of the wild-type strain under similar condition of growth and fermentation time. In another study, a mutant strain of *A. xylinum* BPR 2001 was developed that produced 36% more BC in the agitated-bioreactor compared to the parent strain (Bae et al. 2004b). Nakai et al. (1999) expressed mutant sucrose synthase gene in *G. xylinus* to enhance BC production in an agitated-bioreactor. There have been reports of naturally occurring strains of non-cellulose producing mutants of *G. xylinus* (Forng et al. 1989; Valla and Kjosbakken 1982). Similar non-reversible mutants can be produced by chemical mutation using nitrous acid, nitrosoguanadine and ethyl methane sulfonate. These mutants are useful for BC synthesis related studies.

1.18.2 Growth medium

The medium is the most important criteria for the cell growth and production of BC as the bacteria derive the essential substrates necessary for the same from the medium. The medium can further be sub-divided into the type and quality of substrates besides sugar;

other trace elements are also required in the medium. Additionally, it has also been noticed that addition of certain chemicals/polymers enhances the cell growth and consequently the rate of BC production. The detailed description of the same is given in the sections below.

1.18.3 Type of substrates

The fact that BC can be produced on different substrates was identified in 1886 (Brown). The initial studies on *G. xylinus* were conducted with growth medium containing glucose and sucrose as a carbon source (Brown 1886). Tarr and Hibbert (1931) reported that fructose and mannitol (readily converted to fructose by the bacteria) gave a higher cellulose production rate compared to glucose. Reports of use of D-mannitol have also been published by Minor et al. (1953). Since then there have been many studies using different substrates for BC production. Some conflicting reports have also appeared that stated glucose gave the highest yield of BC compared to other carbon sources (Masaoka et al. 1993; Slusarska et al. 2008), while Embuscado et al. (1994) reported it to be fructose whereas Nakai et al. reported it as sucrose (1999). Oikawa et al. (1995) found that D-mannitol increased the rate of BC production by three times when compared to glucose under ideal conditions. Recently, one such study conducted by Sherif et al. (2005) showed that among the various monosaccharides, disaccharides and alcohols investigated, glycerol gave an improved yield over glucose and fructose. It was also noted that concentration of the sugars also played a significant role. There have been reports on the production of BC on non-defined substrate medium such as pulping waste liquor (Uraki et al. 2002), corn steep liquor (Noro et al. 2004), coconut water (Budhiono et al. 1999) agricultural waste from coconut and pineapples (Kongruang 2008) and molasses (Bae and Shoda 2004).

1.18.4 Substrate concentration

The affect of initial glucose concentration was investigated by Masaoka et al. (1993). They found that the yield of BC per g of glucose consumed ($\text{g}_{\text{BC}}/\text{g}_{\text{glu}}$) was inversely proportional to the concentration of glucose in the medium. This was due to accumulation of gluconic acid especially at high concentrations that lowered the pH. This is because glucose was preferentially converted to gluconic acid over cellulose. In another study comparing different concentrations of substrate by weight, it was found that 1%, 5% and 15% gave better rates of BC production compared to concentrations higher than 15% and utilization of substrate was the best at around 5% concentration (Embuscado et al. 1994).

1.18.5 Additives

BC is commonly produced in the medium developed by Schramm and Hestrin (1954a) but reports on the use of synthetic medium have also been published Son et al. (2003). The earliest known additive that enhanced BC production was ethanol as reported by Tarr and Hilbert (1931), and confirmed by other researchers (Chávez-Pacheco et al. 2005; Krystynowicz et al. 2002; Naritomi et al. 1998; Schramm and Hestrin 1954a). The addition of lactate increased the metabolism of the bacteria thereby increasing the rate of BC production (Naritomi et al. 1998). Additions of different co-polymers, chemicals and/or enzymes have also been known to affect BC production as well. Lapuz et al. (1967) showed that addition of 0.5% ammonium phosphate (range 0.1-1.3%) gave a higher BC production and Tajima et al. (1996) enhanced BC production by 50% by addition of water-soluble chitosan. The addition of endoglucanase, an enzyme isolated from *Bacillus subtilis*, increased cellulose production (Tonouchi et al. 1995). Addition of water-soluble polysaccharides such as acetan and agar are also reported to have improved the rate of BC production (Bae et al. 2004a; Chao et al. 2000; Ishida et al. 2003). In

another study, the addition of polyacrylamide-co-acrylic acid to a fructose based medium resulted in the increase in the BC production (Joseph et al. 2003). The addition of sodium glutamate, a good nitrogen source for *G. xylinus* (Ramana et al. 2000) in glucose and ethanol based medium increased the cell growth by 4 to 5 times, and the cellulose production by 1.8 times in static-culture (Chávez-Pacheco et al. 2005). Recently, an increase in BC production was reported by the addition of 0.04% (w/v) of sodium alginate (Zhou et al. 2007a).

1.18.6 Operating parameters

G. xylinus is an obligate aerobe that requires specific environmental conditions for favourable growth and reproduction. This includes an ambient temperature of approximately 30 °C, a pH between 4-5 and atmospheric oxygen tension of about 20%. The impact of the factors mentioned will be discussed in greater detail in the sections below.

1.18.7. Temperature

Nata production studies found that the favourable temperature for BC production was between 20 and 30 °C (Lapuz et al. 1967). They arrived at this conclusion based on the observations conducted during a 48-hour period after initial inoculation. The cell numbers and their growth were observed at 10, 15, 35 and 40 °C, respectively. A similar study has been reported very recently by El-Saied et al. (2008). They produced BC in static-culture over a temperature range of 20-37 °C. They found the maximum rate of production was obtained at 30 °C and at 37 °C and above no BC was detected. Another recent study evaluated the yield of BC between 25-35 °C and the maximum yield was recorded at 30 °C (Slusarska et al. 2008). The temperature can be controlled with ease in almost all known methods of BC production.

1.18.8 pH

Among the operating parameters, the earliest subject of study was the pH of the culture medium. Different studies unanimously tend to agree that the optimum pH for BC production is between pH 4.0-6.0 with the maximum at pH 5.0-5.5 (Embuscado et al. 1994; Hestrin et al. 1947; Lapuz et al. 1967; Masaoka et al. 1993; Toda et al. 1997; Verschuren et al. 2000). There have been studies that indicate that the growth of *G. xylinus* is inhibited at pH less than 3.5 (Tosic and Walker 1946). Barring the static cultures, pH can be easily controlled in the agitated and rotating bioreactors-based production methods.

1.18.9 Oxygen uptake

Since *G. xylinus* is an aerobic bacterium, aeration is critical for growth and subsequent cellulose production. In the static culture method, aeration or oxygen uptake plays an important part in BC production, although no direct relationship has been observed between the two (Verschuren et al. 2000). They found that oxygen diffusion is more critical in the earlier stages of fermentation. However, excessive oxygen supply reportedly results in a decrease in BC production due to loss of substrate by direct oxidation (Yamanaka 1988). Contrasting reports claim that in case of fructose, an increase in oxygen can enhance the production (Kouda et al. 1997a).

1.18.10 Impact of excessive aeration in agitated-bioreactors

The negative effect of excessive aeration is more pronounced in the agitated culture, which is the most popular commercial method of BC production. Some groups have suggested that normal aeration throughout the growth medium promotes preferential production of non-cellulose producing cells (a phenotype having selective advantage in oxygen-rich condition) over cellulose producing cells (Krystynowicz et al.

2002;Schramm and Hestrin 1954b). Supporting reports about the decline in cellulose-producing cells with a subsequent rise in the non-cellulose producing mutants due to increase in the agitation and consequently aeration was published by Ross et al. (1991). Hestrin and Schramm (1954b) have reported this as the primary cause of loss of BC productivity in agitated culture method. The mutants can be distinguished from the cellulose-producing cells from the morphology of the colonies. The colonies of non-cellulose producing mutants are, in general, flattish and less gelatinous as compared to those of the cellulose producing cells, which are roundish, convex and more gelatinous (Yamada 2000). Among these non-cellulose producing mutants, some have the capacity to revert to cellulose-producing cells under static culture conditions while others do not. While most researchers have pointed to excessive aeration as the reason for these conversion, some have attributed this phenomenon to the inactivation of the gene encoding cellulose synthase, which is capable of shifting its site in the genome (Coucheron 1991).

1.18.11 Impact of oxygen tension

Varying oxygen concentration by changing the partial pressure also affected BC production. It was inhibitory in static cultures when the oxygen tension in the gaseous phase was greater than atmospheric tension (Watanabe and Yamanaka 1995). Whereas, when the oxygen tension was lowered by 10-15% compared to the atmospheric conditions, the BC production increased by 25%. This change in oxygen tension had an impact on the property of the BC produced as well. The BC produced in static cultures under higher oxygen tension was harder in texture compared to that produced at lower oxygen tension. The oxygen tension seems to have a reverse affect in the agitated culture method (Schramm and Hestrin 1954a). They found that maximum BC production was at

100% oxygen tension and it reduced to about half when the oxygen tension was reduced to 20% or normal atmospheric concentration.

1.18.12 Other factors

Some factors influencing the BC production are specific to the method of production that include the agitator configuration (Kouda et al. 1997b; Kouda et al. 1996). Valla and Kjosbakken (1982) reported the addition of antibiotics that block RNA or protein synthesis into the medium containing cellulose non-producing cells reactivated BC production in agitated cultures.

1.19 Applications for bacterial cellulose

Bacterial cellulose is a very unique biopolymer with exceptional properties that include high water holding capacity, hydrophilic in the wet form, high tensile strength and hydrophobic in the dry form. These properties are being investigated rigorously as is evident from an increasing number of papers on applications or their potential in various fields. This has been additionally fuelled by consumer interest in biomaterials.

Almost all the unique properties of BC have been leveraged for commercial applications. Yamanaka and Watanabe (1994) focussed on the high Young's modulus and shape retention ability of the dry BC and developed material for speaker diaphragms for the Sony Co. They also suggested the addition of disintegrated BC to paper pulp in order to produce stronger paper. This application has also been explored for stereo diaphragms (Jonas and Farah 1998). There have been reports of innovative research that proposed the use of BC as an electronic display paper (Shah and Brown 2005) and bio-sensor chip made with optical compact discs and BC fibres (Tabuchi et al. 2005).

The filtration and permeability of BC was investigated by Takai (1994). Polymers such as polyethylene glycol, carboxymethyl cellulose, carboxymethyl chitin and other cellulose-based polymers were incorporated in-situ into the growth medium. He found that the composites produced by this method had lower flux rates compared to plain BC. He suggested the usefulness of this property in ultrafiltration and pervaporation. Another researcher exploited the filtration property of BC to produce a dialysis membrane (Shibazaki et al. 1993). On comparison with the membranes made from regenerated cellulose, they found that the BC membrane had significantly higher permeation rate, greater flexibility in molecular weight cut-off and higher mechanical strength that allowed the use of thinner BC. An example of the application of such a membrane has been dialysis membrane (Sokolnicki et al. 2006; Wan and Millon 2005).

Fontana et al. (1990) focussed on the medical application of BC since it is known to have apyrogenic properties. They conducted clinical trials on burns and other skin injuries using never-dried BC sheets called Biofill[®]. They reported positive results including relief from pain, hastened healing and good exudates retention. Similar reports of artificial skin have been made by other researchers (Czaja et al. 2007; Jonas and Farah 1998). Another group focussed on the medical application and utilized the mouldability of the BC to produce Bacterial SYnthesized Cellulose (BASYC)[®] (Klemm et al. 2001). The high mechanical strength and water retention in the wet form and the smooth inner surface fulfilled the requirement for use as artificial blood vessels during microsurgery. Various researchers have investigated medical applications and have suggested a wide range of applications including wound dressing (Ciechańska 2004; Czaja et al. 2007; Wan and Millon 2005), dental implants, vascular grafts, catheter covering dressing, coatings for cardiovascular stents and cranial stents (Czaja et al. 2007; Wan and Millon 2005),

membranes for tissue-guided regeneration, tissue replacement, controlled-drug release carriers, vascular prosthetic devices and scaffold for tissue engineering (Czaja et al. 2007), and as artificial blood vessels (Klemm et al. 2001; Wan and Millon 2005).

The Weyerhaeuser Co. (Tacoma, Washington, USA) and Cetus Co. (Emeryville, California, USA) were the first to produce BC on a large scale leading to the development of Cellulon[®] a broad spectrum bulking agent. Okiyama (1992a) suggested treatment processes using ethanol or alginate along with calcium chloride in order to make the BC more edible.

1.20 Modified bacterial cellulose

Recently, there has been a great surge in the use of bio-composites because of their biodegradability. This has propelled researchers to combine different soluble polymers with BC to produce novel composites. Some groups have achieved this addition of different miscible polymers into the medium during production so that it gets entrained into the BC (Chanliaud and Gidley 1999; Ciechańska 2004; Ifuku et al. 2007; Ogawa and Tokura 1992; Seifert et al. 2004; Whitney et al. 1999; Yano et al. 2008). When the polymer is added in the medium, it can co-crystallize with BC fibrils to produce thoroughly blended composite material (Benziman et al. 1980). Depending on the properties and concentration of the polymer used, the physio-mechanical properties of the BC can be impacted.

Some groups have added different solids during production for physical entrapment in order to change properties of the BC produced without affecting the crystallite structure (Mormino and Bungay 2003; Serafica et al. 2002). While others have preferred to treat the BC (never dried or dried form) with some chemicals and/or co-polymers post production

(Barud et al. 2008;Evans et al. 2003;Gindl and Keckes 2004;Hussein et al. 2005;Ifuku et al. 2007;Maria et al. 2010;Phisalaphong et al. 2008a;Seves et al. 2001;Svensson et al. 2005;Wan and Millon 2005;Yano et al. 2005). Irrespective of the method all these researchers produced novel materials with properties different compared to that of pure BC. Many of the researchers have patented their novel products and /or methods. A list of some such published patents is given in Appendix 1. This list also includes novel strains, methods and uses of BC.

1.21 Biotechnology perspective of bacterial cellulose

Although bacterial cellulose with its unique properties holds much promise in the industrial field, it has to overcome a number of hurdles to claim its a place in the commercial world. Some of the main commercial drawbacks are that the production methods, such as static-culture, are not economically viable, while the genetic instability of the *Gluconacetobacter spp.* hinders production efficiency in agitated-bioreactors, thus the development of an ideal and economical method of BC production is quite critical. A fair amount of work is being reported on the genetic engineering of high cellulose producing strains of *G. xylinus* but more work is required to tackle the issue of genetic instability. Once these keys issues are resolved, the knowledge of the affect of various environmental parameters, addition of various materials and post-production treatments can be applied to utilize BC to its maximum capacity.

CHAPTER 2

Materials and Methods

2.1 Microorganism

The culture of *Gluconacetobacter xylinus* ICMP 15569 was used for all the fermentation runs. This strain was isolated by our group from kombucha tea (Holmes 2004). As this strain was present in the New Zealand environment, less environmental handling restrictions were imposed by the Hazardous Substance and New Organism Act 1996 compared to an imported strain.

2.2 Culture conditions

The bacterial cultures have been cultivated and maintained in our group since their isolation in 2004. The cultures were incubated at 30°C in 86 mm x 27 mm McCartney glass bottles, filled with 15 ml of growth medium. New cultures were inoculated on a regular basis using 1 ml of previous cultures (7-8 days old) tubes. The medium for a new rotating-bioreactor run was inoculated with 15 ml of the culture (3 days old). The inoculation was conducted in a Clyde-Apac® Clean Air BH2000 laminar air-flow biological cabinet. The average cell density of the inoculums was, 4×10^5 cells/ml, as estimated using haemocytometer under the microscope.

2.3 Agar Plates

In addition to suspended cultures, plates of agar were prepared aseptically with glucose medium containing 4% agar. Solo colonies were transferred to fresh plates on a fortnightly basis. The colonies were streaked onto plates regularly to ensure the purity of the culture (Fig. 2.1). If the culture was contaminated or there was a change in phenotype, it showed more prominently in the agar plates. A typical streak with pure culture on an

agar plates would form colonies in 48 hours. They colonies would be round, small and appear translucent at first and after 72 hours would turn whitish in colour. The contaminated cultures would not form colonies on streaking even after 72 hours. Sometimes colonies that were bigger flattish and grey in colour would form showing contamination, although these colonies were observed in less than 2% of the cultures.

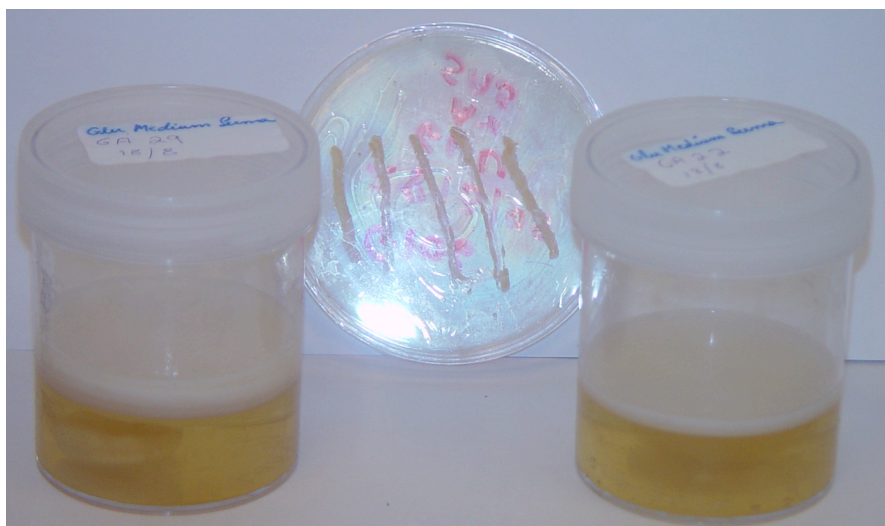


Fig. 2.1 – BC produced by the static culture method at the air liquid interface in polypropylene bottles (100 ml, 22 mm) and *G. xylinus* colonies streaked on agar plate in the centre.

2.4 Storage of culture and BC pellicles

As a precautionary measure in case of contamination cultures, *G. xylinus* cultures were frozen in microtubes. Each microtube contained 200 μL of active broth along with 800 μL of pure glycerine. After careful mixing, the microtube was stored for 20 minutes at room temperature. For short period storage, the microtube was stored at -18°C . For longer duration storage, the microtube was transferred after 20 minutes from -18°C to -78°C . In order to re-establish a backup, the culture was streaked onto an agar plate or tubes with medium after thawing.

2.5 Growth Medium

The medium for growth used for all fermentations was a modified version of the medium used by Serafica et al. (2002). Every 1 L of growth medium contained glucose 50 g, ammonium sulfate 5 g, sodium phosphate (dibasic) 2.7 g, magnesium sulfate 1 g, yeast extract (Sigma) 0.5 g, citric acid 1.5 g, ethanol 14 ml and 2 ml of trace element solution adapted from Mormino (2001). The trace element solution was made up of EDTA tetrasodium salt 570 mg, $\text{FeSO}_4 + 7 \text{H}_2\text{O}$ 200 mg, $\text{ZnSO}_4 + 7 \text{H}_2\text{O}$ 10 mg, $\text{MnSO}_4 + 4 \text{H}_2\text{O}$ 34 mg, H_3BO_3 30 mg, $\text{CoCl}_2 + 6 \text{H}_2\text{O}$ 30 mg, $\text{NiCl}_2 + 6 \text{H}_2\text{O}$ 3.6 mg, $(\text{NH}_4)_6\text{Mo}_7\text{O}_{14} + 4 \text{H}_2\text{O}$ 2.4 mg per litre. All chemicals were used as received.

2.6 Co-polymers

Different co-polymers such as gelatin, chitosan and chondroitin sulphate were added into the growth medium to produce modified BC. Gelatin (Fluka) and Chitosan (from shrimp shells) was obtained from Sigma Aldrich, Auckland, New Zealand. The chondroitin sulphate (animal origin) was ordered from New Zealand Pharmaceuticals, Palmerston North New Zealand. Since gelatin was easily miscible, it was directly dissolved into the growth medium. Chondroitin sulphate was dissolved in deionized water and stirred for 1 hour and chitosan was dissolved in 1% acetic acid prior to mixing with the medium. The medium was autoclaved after the co-polymers were mixed in it. All chemicals were used as received.

2.7 Static culture method

BC was produced in static cultures in sterilized, polypropylene, wide-mouth bottles of two different sizes (100 ml / 32 mm ID and 200 ml / 45 mm ID). The medium was similar to that used in the rotating-bioreactor, and were inoculated using 1 ml medium from three-day-old cultures in the McCartney bottles. These bottles were then left undisturbed

in an incubator maintained at 30 °C with the lids loosely placed on them. The cellulose pellicle appeared at the air medium interface after 48-72 hours. The date of appearance of the pellicle was recorded and used in the calculation for rate of production. The medium samples (1 ml) were taken on the day of inoculation and at harvest to determine the glucose concentration for the calculation of yield. The initial amount of medium and the pH at the time of inoculation and at harvest was also recorded. The method of harvest and storage is similar to that of the rotating-bioreactor.

2.8 Rotating biological contactor (rotating-bioreactor)

The rotating-bioreactor used for all the fermentation runs was designed by Schrecker (2005). It was made up of two stainless steel vessels; one was a fermentation vessel with rotating cylinders and another, a medium reservoir (Fig. 2.2). An ASEA 0.18 kW motor transferred torque to the cylinders via a Haitec 1:60 gearbox and a magnetic coupling. The rotational speed of the cylinders was controlled as desired by a RRF Fisher AC controller. The *G. xylinus* grew on the outer surface of two cylinders. A small cylinder (C_S) and bigger cylinder (C_L) of diameter 120 and 140 mm respectively and length of 120 mm (Fig. 2.2) were used. Both the cylinders were covered with felt material to provide for cell attachment. The submersion level cylinders in the medium were maintained at 26% for C_L and 21% for C_S for all the runs. The stainless steel fermentation vessel of dimensions (length x height x width: 365 × 190 × 182 mm) was sealed with a clear polycarbonate lid (10 mm thickness) with openings through which the tubing were connected. The stainless steel, reservoir vessel had similar dimensions, except the height was 90 mm.

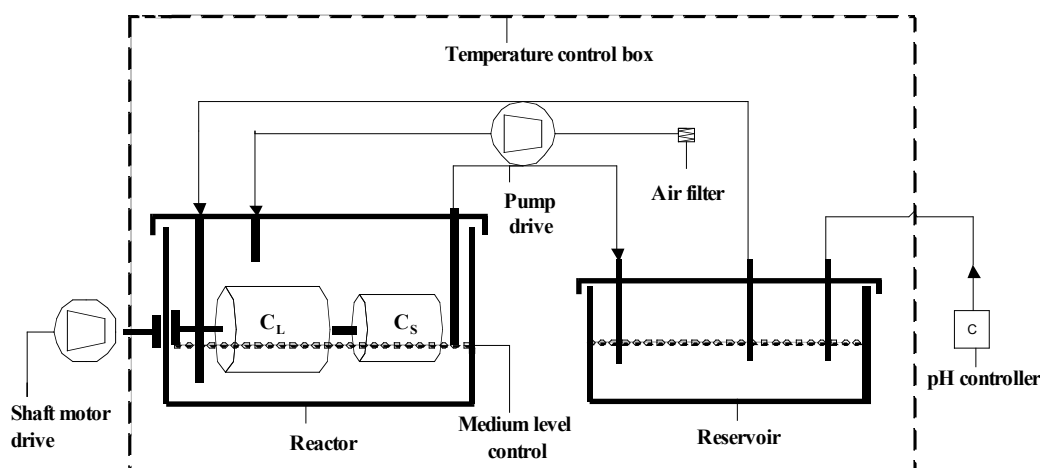


Fig. 2.2 – A diagram of the rotating biological contactor (rotating-bioreactor).

2.9 Control of medium level

The growth medium was continuously circulated between the fermentor and the reservoir using a multihead, Master flex L/S console drive (Cole-Parmer®). The level control was maintained by adjusting the height of the fermentor's outlet tubing to the required level. This level control was required because as cellulose formed on the cylinders, the medium level in the reactor would fall due to liquid being suspended in the wet cellulose as it rotated out of the solution. Therefore, the reservoir maintained the level in the reactor throughout the duration of the run at the cost of its medium level. The medium circulation during level control also supplied additional mixing in both vessels. Norprene® tubing of different diameters 6404-17 and 6404-18 provided a higher pumping rate from the reactor to the reservoir. To achieve a good mixing of the medium, the reservoir vessel was stirred constantly with a magnetic stir bar.

2.10 pH control

The pH was measured by an Orion 9104SC pH electrode placed in the reservoir connected to an Intech® pH amplifier. To reduce electronic noise picked up by the pH

electrode, the reservoir vessel was grounded. A Shimaden® PID controller controlled a Masterflex® L/S console drive (Cole-Parmer®) (4-20 mA signal) for caustic addition. An Ismatec Pharmed tubing (orange/yellow, ID= 0.85 mm) and 0.25 M NaOH were used to maintain the desired pH. The rate of caustic addition and pH were recorded using a data logger (Trutrack®). In order to reduce noise, a 100 seconds averaging digital filter was laid on the pH signal. The average of the reservoir pH value and caustic pump rate over 6 minutes was logged with a separate Trutrack® data logger.

2.11 Aeration and temperature control

The rotating-bioreactor and reservoir were enclosed in a temperature control box made of polystyrene. They were maintained at 30 °C with a light bulb as the source of heat and temperature controller (Digi-Sense, Cole-Parmer®). Air was bubbled aseptically into the reactor at 64 ml/min through an air filter, using a peristaltic pump using 6404-16 Norprene® tubing. Two 0.45 µm Millipore filters were used for exhaust in both the vessels.

2.12 Setup

Three days prior to a run, a McCartney tube was inoculated as a pre-culture. Eight litres of glucose medium was autoclaved and cooled before the run. All components of the reactor were washed with dish washing detergent, rinsed in deionised water and 70% alcohol and then autoclaved for 30 minutes at 121 °C. The reactor and the reservoir along with its components were assembled in the biological cabinet. The trace element solution was added into the medium and aseptically poured into the reactor and reservoir. The medium outlet of the reactor was adjusted to the desired medium level. The pre-culture was transferred aseptically into the reactor and sealed.

The rotating-bioreactor was then moved to the table with the temperature control box where the pumps, pH probe (cleaned with 70% ethanol), temperature probe, caustic and air supply and grounding for the reservoir were installed. The initial pH level was usually between 4.9 and 5.2. It was adjusted to the desired level at the beginning of all runs in which the variation of pH was investigated (pH 3.0-6.0) except for those controlled at pH 4. The desired RPM was maintained by adjusting the motor of the shaft rotating the cylinders and was reported as tangential velocity. In runs that included co-polymers, the desired co-polymer was blended into the medium itself after autoclaving it separately.

2.13 Operation

During the course of the run, 5 mL samples were drawn from the reactor everyday at approximately the same time. Out of the 5 ml of each sample, 4 ml was used for pH analysis while 1mL was filtered and stored at -18 °C for future use to determine glucose concentration and acid content. The level control system was checked for blockages daily. The data from the pH data logger was downloaded every day. A pH drop was an indication of the beginning of exponential growth and its time was recorded. The caustic pump was turned on at the beginning of the run so that the pH was maintained at the desired level throughout the run.

2.14 Harvest

A normal fermentation lasted 5-10 days after the initial pH drop before cellulose was harvested. Sometimes the BC formed in places other than the cylinders and was removed carefully with tweezers to minimise clogging in the recirculation tubing. This was saved and taken into account during the final analysis. Once the run was complete, the tubes and pH probe were disconnected while the cylinders kept rotating. The pellicles were harvested from the cylinders swiftly using a sharp blade and weighed before transferring

them into a tub of deionised water. The weight of the pellicles was used for estimating the rate of production and yield. The BC that was formed in the reservoir and reactor surfaces, other than on the cylinders was collected, weighed and then autoclaved and air-dried. The harvested pellicles were boiled in 0.5 M NaOH solution for 15 minutes to remove *G. xylinus* cells and remnant medium. They were then rinsed twice and soaked in deionised water (DIW) for 48 hours before being used for further analysis.

2.15 Storage of BC samples

The BC samples were stored in plastic tubs with 0.2% (w/v) sodium azide solution in deionised water to protect the samples against microbial and fungal contamination. When sodium azide was used to store unclean BC samples (without boiling in caustic) for analysis, it was important to buffer the solution at pH 7 as volatile hydrazoic acid (HN_3) can be formed at the low pH values associated with cellulose production. This acid is very harmful if inhaled at significant concentrations.

2.16 Post production Assays

2.16.1 Glucose assay

Glucose concentration was measured offline using YSI 2700 SELECT biochemistry analyser (Yellow Springs Instrument Co., Inc.) at the end of each fermentation run. The 1.5 ml frozen medium samples were thawed prior to analysis. The instrument was calibrated with a 2.5 g/L glucose standard at the beginning of each analysis. The glucose concentration recorded was used to calculate the yield.

2.16.2 Acid determination using High Performance Liquid Chromatography (HPLC)

HPLC was used to determine the concentration of acetic and gluconic acids produced in the medium samples taken during fermentation runs. The Hewlett Packard 1100 Series

(Hewlett-Packard Development Company, L.P), equipped with a Hewlett Packard 1047, a refractive index detector was used. The column used for the separation was an Econosphere C18 5 μ , ID= 4.6 mm, and length 250 mm. A guard column was used to protect this column. The mobile phase was 1% phosphoric acid: acetonitrile 95:5. In this study, the acids were detected and quantified using refractiev index detector (RI), but the elution was also monitored with UV at 210 nm. The flow rate was 1.0 ml/min and injection volume of sample 0.1 μ l. The upper pressure limit was 170 bars. The retention time for gluconic acid and acetic acid was 2.65 and 3.31 min respectively.

2.16.3 Water holding capacity (WHC)

The water holding capacity (WHC) was determined using the method developed by Schrecker and Gostomski (2005). Using a punch wad ID=25 mm, 5 samples per run per pellicle were cut. The samples were then stabilised under 10 mm vacum for four hours before determining the wet weight (Fig. 2.3). The samples were air dried before oven drying at 104 °C for 12 hours to give the dry weight. The WHC was calculated (water mass / dry cellulose mass) for each of the samples and the average WHC was calculated and reported. The wet and dry weights of the samples were determined with a Mettler AB204 balance (0.1 mg resolution).

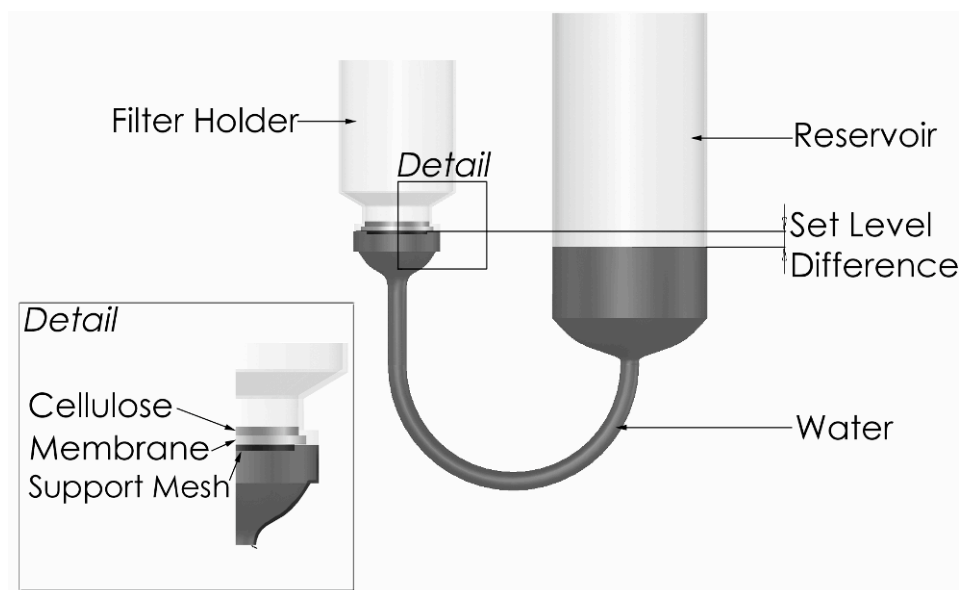


Fig. 2.3 - The set up for determining the wet weight of the wet BC samples using vacuum method. Adapted from Schrecker (2004).

2.16.4 Rewetting experiment

The experimental design was similar to that used in the WHC assays. The only difference was that instead of a hanging column, a longer burette was used that was connected to the filter via transparent tubing Norprene® 64004-16. The water level in the reservoir and tubing was lowered down to several heights in order to maintain different water tensions. The samples were prepared in the same manner as in WHC assays and were held under different values for tension for similar length of time (4 hours) and weighed. The samples were then immersed in DIW for different length of time for rewetting. After rewetting the same procedure of finding out the WHC was repeated.

2.16.5 Scanning electron microscopy (SEM)

An SEM microscope Leica S440 (Leica Microsystems Wetzlar, Germany) was used. The wet samples of BC were frozen in liquid nitrogen and freeze fractured with the help of tweezers. The samples were then mounted using a carbon tab and covered with

conducting carbon paint to observe the fractured surface area. A Polaron 5000 sputter coated the sample under the following conditions: 20 mA at 1.2 kV for 4 minutes using nitrogen as the distributing gas. The specimens were observed at 5 KeV/50 pA at standard magnifications in all cases. A micron scale on the micrographs indicates the actual magnification.

2.16.6 Confocal scanning laser microscopy (CSLM)

The BC samples were observed using Confocal scanning laser microscope Leica TCS SP5 (Leica Microsystems Wetzlar, Germany). Samples of size approximately 5 mm² were cut from freshly harvested and untreated BC. The samples were stained with the dye Rhodamine blue of appropriate concentration (10 ppm) for 10 minutes and the excess dye was washed under running tap water. The samples were observed under a laser scanning beam at excitation wavelength 590 nm and emission wavelength of 633 nm. The images were recorded with the LAS AF version 1.7.0 software. The images were collected from the BC samples using time series in focal slices of 0.6 µm each.

2.16.7 Image analysis

All the images were analysed using the Image-Pro software version 4 (Media Cybernetics). The measurement of the width of the BC ribbons was conducted using the micrographs of the images taken in the SEM and analysed using the measurement tool of the Image-Pro software. The width of the ribbons was measured at the widest part (Yamanaka et al. 2000). About 30 ribbons were measured from 5 samples for each of the pellicles produced in the static-culture and all the pellicles produced at different pH and no pH control in the rotating-bioreactor.

The images of the bacteria taken from the CSLM were bright in colour against the dark background and were quantified using the tool to count the number of bright objects. A

series of images were collected at a depth of 100 micron each from both sides of the pellicle in slices of 0.6 μm each. The images were stacked together using the Leica software the number of bacteria calculated on Image-Pro software. The sample size was three different observation sites from each sample and 10 different samples were taken from the pellicles produced in the static-culture and the rotating bio-reactor at pH 4 and no pH control.

2.16.8 Mechanical testing

2.16.8.1 Mechanical testing of BC using Material Testing System (MTS)

The mechanical tensile testing of dry, BC samples was conducted on a Material Testing System model MTS-858 table-top system (MTS® Systems Corporation). A load cell of 2.5 kN was used. The samples were cut to (70 × 30 mm) and dried for 24 hours at 104 °C and stored in plastic bottles containing silicon crystals. The sample thickness was recorded using a micrometer. The samples were gripped pneumatically at 15 PSI. The test rate of the samples was 0.1 mm/sec. The readings were taken only from the samples that failed at approximately at the midpoint from both the grips.

2.16.8.2 Compression testing using Instron

The compression tests were conducted on the Instron universal testing machine model-1011 (Instron. Corp). A custom-made confined compression chamber made up of stainless-steel with an inner diameter of 25 mm and depth of 10 mm was used (Fig. 2.4). The platen used for compression was 10 mm in thickness, 22 mm diameter and had a porous material made up of sintered brass (4 mm) at the bottom to allow the water to escape from the samples without impacting the stress. Ten samples of diameter 14 mm were cut under water using a punch wad from each of the BC samples under investigation. The samples were stored in deionised water until test. The compression

chamber was placed in a polycarbonate bottle (400 ml / 90 mm ID) filled with deionised and degassed water. The sonicated platen was attached to the clamp while under water and aligned with the compression chamber. The load cell was adjusted to zero and a sample was placed in the compression chamber.

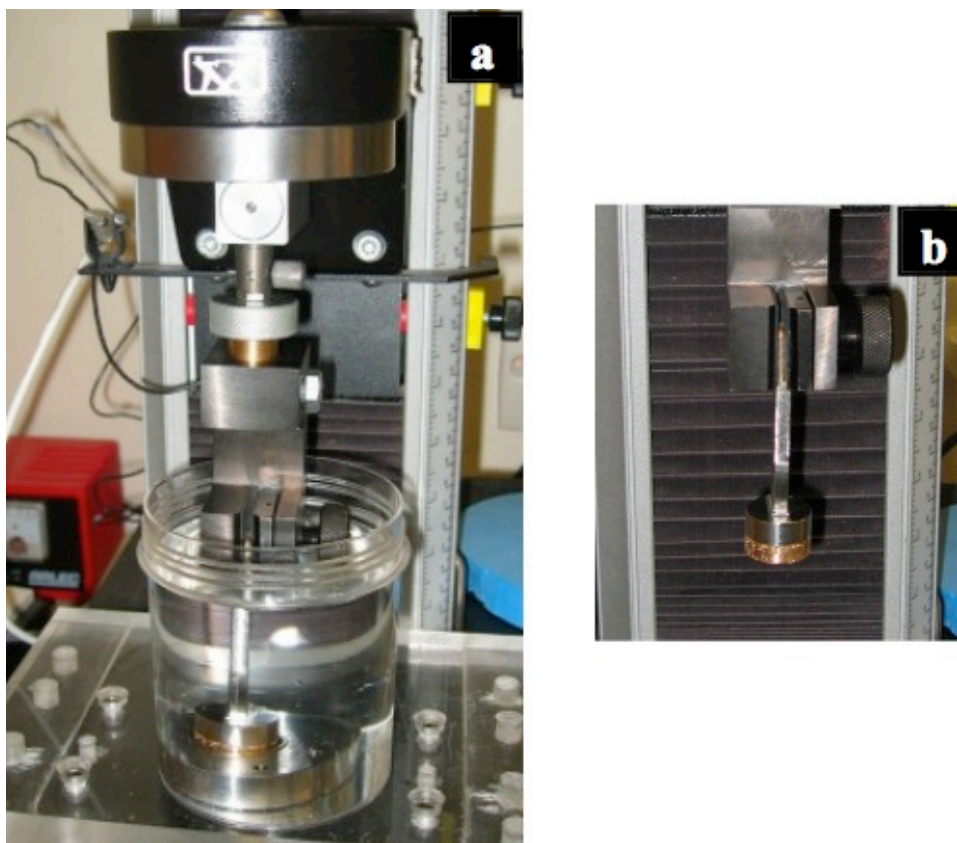


Fig. 2.4 – The custom made compression chamber and platen submerged under water during the compression test Fig.2.4-a on the left and the platen made up of steel and porous, sintered brass on the right Fig.2.4-b (the reflection of light from the brass does not show the pores clearly).

Compression tests were carried out with water but without the samples to find the force exerted by pure water (blank reading). The actual values obtained for each sample were corrected for buoyancy effects by subtracting the blank reading. The reported results were modified accordingly. The platen was lowered onto the samples at different speeds

(0.1, 1 and 10 mm) and the force vs displacement profiles were recorded. It was found that at the speed of 10 mm / sec a more regular force vs displacement profile with lesser variation was obtained. For all the samples the speed of lowering the platen was maintained constant at 10 mm / sec. The platen was lowered on the samples until the maximum force was attained. The Firmness Program for Instron was used to access the maximum force exerted by the samples until failure. A 50 N load cell was used for all the samples produced in the rotating-reactor and a 500 N load cell was used for samples produced in static-bioreactor.

2.16.9 X-ray diffraction

The X-ray diffraction was performed on oven-dried BC from different fermentations runs. The X-ray diffraction was conducted using MoK α radiation ($\lambda = 0.71013 \text{ \AA}$) and data collected with a Bruker CCD\SMART area detector. The X-ray patterns were obtained with Philips PW1729 diffractometer using Cu K α radiation ($\lambda = 0.1540 \text{ nm}$), a voltage of 50 kV and current of 40 mA with 2θ increased in steps of 0.02° . The adjacent averaging smoothing function in Origin $^{\text{®}}$ Pro 7.5 was used to smoothen out 30 adjacent data points from the x-ray raw data and plots were then normalized for area.

2.16.10 Data analysis

The statistical analysis was carried out using the software GraphPad Instat version 3.0 for Macintosh. The statistical significance of the evaluated data was analyzed by one-way analysis of variance (ANOVA) and unpaired T-test. Differences among the mean values were tested using the least significant multiple range test. The values were considered significant when $p < 0.05$. The error analysis is reported as the standard deviation of the mean and the varying sample size for different treatments is mentioned.

CHAPTER 3

Structural properties of BC produced under different conditions

3.1 Introduction

The BC is a complex 3-D mesh-like structure made up of random network of highly ordered cellulose ribbons with an average mesh size of about $0.8 \pm 0.56 \mu\text{m}$ (Grande et al. 2008). The culture medium and the method of production is known to have an impact on the morphology of BC produced (Dudman 1959; Hestrin and Schramm 1954b; Marx-Figini and Pion 1974; Watanabe et al. 1998b). The morphology of the BC structure includes the dimensions of the BC ribbons, its sub-unit microfibrils and their arrangement. Any structural changes in either the network or the individual structure and size of the ribbons can affect the porosity of the BC pellicle, which in turn impacts the properties such as water holding capacity and mechanical strength of the BC in both the wet and dry states. These properties determine its application or end-use of the BC produced. Therefore, the study of the factors influencing the properties of BC produced is very important from an industrial point-of-view.

In literature there are many comparative studies on the properties of BC produced by the static-culture and the agitated-bioreactor methods of BC production (Hestrin and Schramm 1954a; Watanabe et al. 1998b). There are fewer comparative studies published on the physical properties of BC produced using the rotating-bioreactor (Serafica 1997). In the following sections, the morphology of the BC produced in a rotating-bioreactor in varying conditions will be compared with that produced in a static-culture. The comparative analysis between the BC produced under different growth conditions using rotating-bioreactor and static-culture will also be conducted. The different growth factors include varying tangential velocity in the rotating-bioreactor, and the pH and initial

glucose concentration in both the static-culture and rotating-bioreactor. For ease of comparison between samples from the static-culture and the rotating-bioreactor, BC samples from the rotating-bioreactor of representative conditions will be used for specific analysis such as X-ray diffractometry.

3.2 BC produced at the air-medium interface

The BC pellicle is produced by *G. xylinus* at the air-liquid interface and new layers are added at the upper portion while the lower portion gets pushed into the medium (Borzani and Desouza 1995; Iguchi et al. 2000; Klemm et al. 2001; Schramm and Hestrin 1954a). Klemm et al.(2001) demonstrated this with the help of strings placed on the upper surface of the pellicle on consecutive days. They found that new layers were formed above the strings. This proved that the new layers were added on the upper layer of the pellicle at the air-liquid interface. A similar experiment was conducted in the current study in collaboration with Haiyuan Piao from the Department of Mechanical Engineering, University of Canterbury. An aseptic non-woven piece of cloth (the square textured material), soaked in growth medium, was placed on a developing pellicle, the new BC layers formed above it (Fig. 3.1).



Fig. 3.1 - The non-woven cloth lying between the BC layers.

Klemm et al. (2001) noted that the cells under the pellicle were dormant but capable of BC production in the right conditions. This was also observed by other researchers (Budhiono et al. 1999; Yamanaka et al. 1989). Similar observations were made in the current study. The medium under the pellicle was used as pre-inoculum to make new sub-cultures in fresh medium for future use as an inoculum.

3.3 Scarring in BC

BC samples were freeze-dried and observed under SEM. It was observed that some parts of the cellulose network that did not appear like ribbons and formed irregular shaped patches or sheets of BC fibrils and/or ribbons of cellulose. This morphological irregularity is called scarring (Schrecker 2004). Scarring occurs when a few BC fibrils and/or ribbons melt into each other (Fig. 3.2). The BC samples from the static-culture showed greater scarring than the samples from the rotating-bioreactor (Fig. 3.3 and 3.4). It was observed that BC samples produced in the rotating-bioreactor showed greater scarring at higher level of water draining while air-drying the samples post-harvest

(Schrecker 2004). Thus, loss of water from BC structure could be one of the reasons for scarring. It has been reported in the past that freeze-drying increases the aggregation of the free BC ribbons network structure, partially leading to a tighter network of BC (Clasen et al. 2006). Further investigation would be required to find out if scarring is an artifact of freeze-drying conducted for SEM observations, or if it is present in never-dried BC samples as well. Studies of BC under hydrated conditions using an environmental scanning electron microscope (ESEM) should be conducted to confirm the same. This study is important, not only from morphological, but also an application point-of-view because such structural irregularities can influence the water holding capacity and physio-mechanical properties, including gas permeability (Clasen et al. 2006).

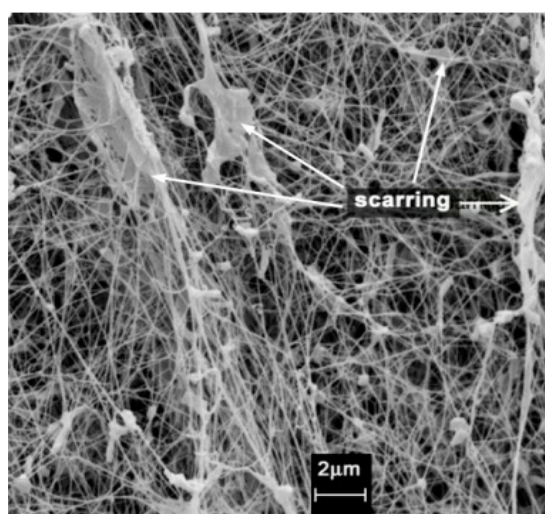


Fig. 3.2 – The SEM micrograph of a freeze-dried sample of BC produced in a rotating-bioreactor showing examples of scarring.

3.4 Morphological and micro-structural study of BC produced in the rotating-bioreactor and static-culture

The BC produced in the static-culture can be distinguished macroscopically from BC produced in the rotating-bioreactor by the difference in appearance and texture. The BC produced in a rotating-bioreactor is less dense, transparent and jelly-like to touch and drains water easily during handling (Fig. 3.3). In contrast, the BC produced in the static-culture is denser, appears whiter in colour and leathery to touch and does not drain water easily during handling (Fig. 3.4). The never dried or wet samples of BC produced in both the static-culture and the rotating-bioreactor were freeze-dried and observed under the SEM. The SEM micrographs of the BC produced in both methods showed a reticulated structure made up of haphazardly inter-woven microfibrils (> 4 nm) and cellulose ribbons of varying thickness (40-60 nm). Although structurally similar, the BC produced in the static-culture was denser and had less space in between the cellulose ribbons (Fig. 3.4-b) compared to the BC produced in the rotating-bioreactor (Fig. 3.3-d). Additionally, the SEM micrographs showed that the BC produced in static culture consisted of a series of dense mat-like layers of cellulose ribbons inter-connected by lower density fibrils (Fig. 3.5). More scarring was observed in samples produced in the static-culture compared to the rotating-bioreactor.

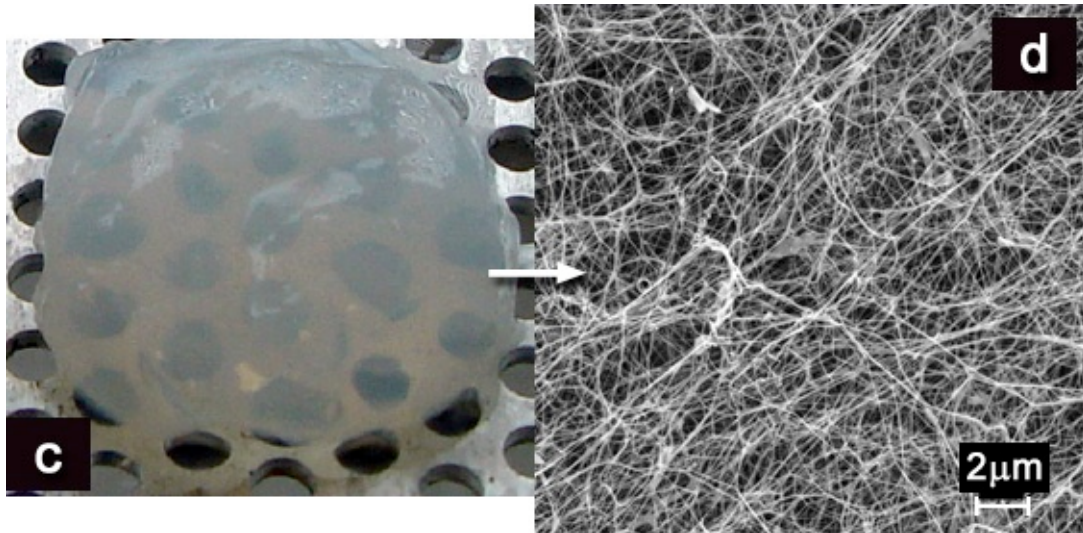


Fig. 3.3 – A snapshot of a BC sample produced in a rotating-bioreactor (Fig. 3.3.c) and the SEM micrograph of the same freeze-dried sample (Fig. 3.3.d).

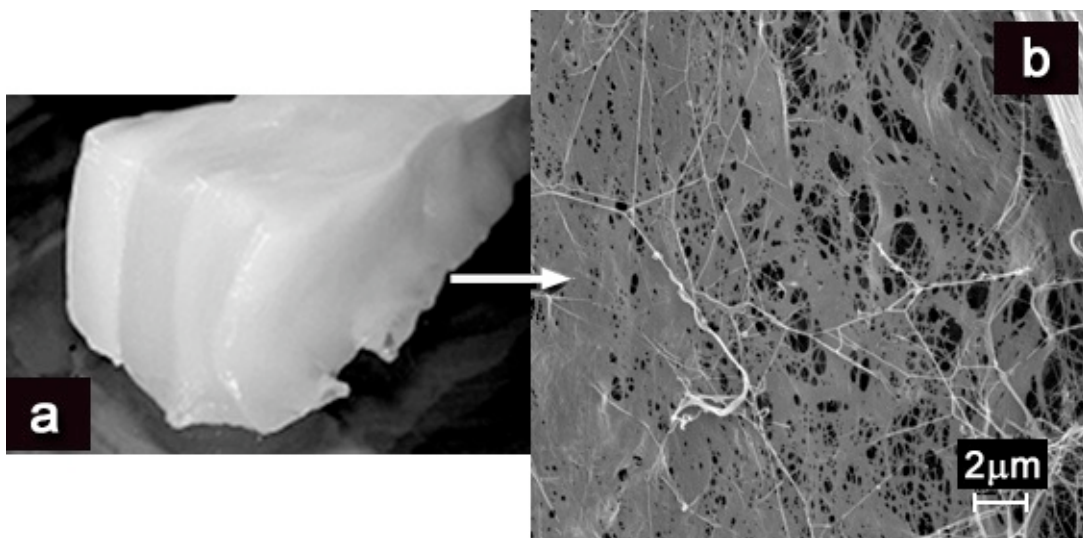


Fig. 3.4 – A snapshot of wet BC produced in static-culture (Fig. 3.4.a) and the SEM micrograph of the same freeze-dried sample (Fig. 3.4.b).

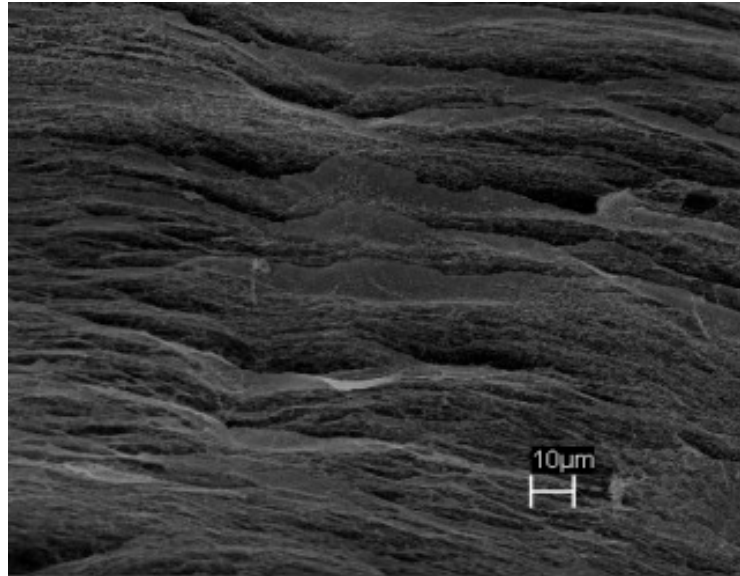


Fig. 3.5 – The SEM micrograph of the cross-section of a freeze-dried sample of BC produced in the static-culture.

3.4.1 Discussion

Our structural findings using SEM are similar to those reported by Serafica (1997), where they found that the BC produced in the rotating-bioreactor appeared like a meshwork of loosely woven BC fibrils compared to the more densely woven BC fibrils in a static-culture. Similar observations of thicker ribbons in static-culture samples in comparison with the rotating-bioreactor samples was reported by Kinsey et al. (2005). It has been reported in the past that gravity does not influence the orientation of the microfibrillar arrangement of the BC produced in static-culture. This was demonstrated by Putra et al. (2008a) by producing BC in silicon tubing in both vertical and horizontal direction. These experiments also indicated the bacteria's preference to move along the longitudinal axis of the tube instead of perpendicular or circular movement around the inner surface of the tube.

More studies investigating the BC production in the agitated-bioreactor and static culture have been published in the past as compared to the rotating-bioreactor. Hence, there are more reports in the literature comparing the structure of BC produced in the agitated-bioreactor and the static-culture than the rotating-bioreactor. BC produced in static culture forms uniaxially oriented overlapping cellulose ribbons (Czaja et al. 2004;Jonas and Farah 1998), while BC cultivated in an agitated culture or in the rotating-bioreactor forms multiaxial fibrous strands (Serafica 1997;Watanabe et al. 1998b). The SEM micrographs published by Czaja et al. (2004) of wet BC produced in both static-culture (thicker fibrils and lesser inter-space) and agitated-bioreactor (finer fibrils with greater inter-space) are similar to the micrographs of samples from static-culture and the rotating-bioreactor respectively in the current study. None of the BC produced under both reactor conditions showed any tunnel structures reported by Thompson et al. (1988) in either wet or dry form.

3.5 Macro-layers in BC

Macro-layers were defined as layers of BC observed macroscopically in never-dried BC pellicles that could be separated manually with ease (Fig. 3.6). These layers were oriented parallel to the air-medium interface or growing end of the BC pellicle. These layers were easily observed in BC produced in the static culture and could be peeled like an onion (Fig. 3.6). The number (5-15) and thickness (2-10 mm) of these layers varied between samples. The BC produced in the rotating-bioreactor at different tangential velocities (0.013 to 0.16 m/s) and varying pH (3 to 6) did not show any such layers on visual observation. However, the BC produced without active pH control (no pH control) showed the presence of these macroscopic layers that could be easily seen and also separated manually (Fig. 3.7). The macro-layers were less thick (1-3mm) and fewer in

number (3-4) when produced in a rotating-bioreactor compared to those produced in a static culture.

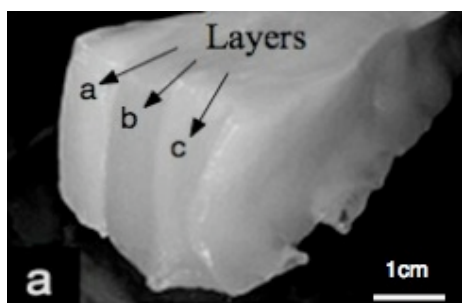


Fig. 3.6 – Wet BC produced in a static-culture showing three prominent macro-layers distinguishable by the different shades of grey, (a) top layer, (b) middle layer and (c) bottom layer.

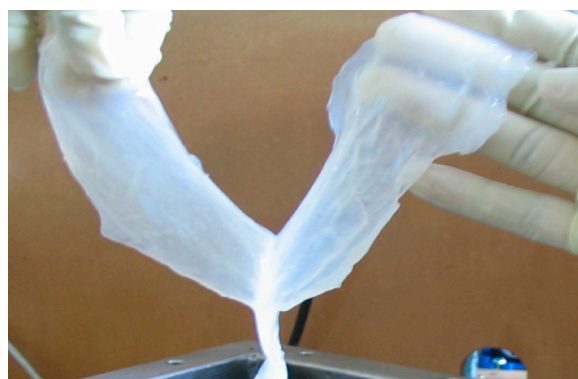


Fig. 3.7 – The manual separation of the BC macro-layers produced in a rotating-bioreactor without pH control.

3.5.1 Discussion

The presence of the macro-layers was reported in pellicles produced in the static-culture by Fontana et al. (1990) and Klemm et al. (2001). While the formation of layers was observed by many other researchers, none of these reports offered any hypothesis for the layer formation in BC pellicles. A probable reason for the formation of layers in static-

cultures could be due to the preferential movement of the bacteria along the axis of the plane of growing pellicle as opposed to perpendicular (Putra et al. 2008a). Since the movement of the bacteria and the BC ribbon formation are interdependent processes (Brown et al. 1976) the layering in static cultures could be a result of the unrestricted bacterial movement parallel to the plane of the growing pellicle. The absence of these layers in BC produced by the rotating-bioreactor could be due to the disruption in the bacterial movement caused by the agitation of the medium in the rotating-bioreactor. The macro-layers in BC pellicles formed in a rotating-bioreactor when the pH was not controlled were similar to the ones formed in the static-cultures. This has been reported in the current study for the first time. There are no published reports about the same in literature.

The current research suggested some role for pH in the macro-layer formation, since similar growth conditions were maintained in the rotating-bioreactor and the static culture. The macro-layer formation (Fig. 3.7) was observed in the rotating-bioreactor only when the pH was not controlled and allowed to drop normally, from \approx pH 4.5 ± 0.5 to 2.45 ± 0.3 . This pH profile is similar to the BC produced in a static-culture, where the pH was not controlled. Whether the pH was the only factor or one of the contributing factors, for the macro-layer formation was not established. One study reports the formation of macro-layers when produced without pH control in the aerosol reactor : the bioreactor in which the substrate is directly sprayed on to the growing pellicle (Hornung et al. 2007). About 3-4 cm macro-layers were achieved by interrupting the substrate supply at intervals of six hours. It has not been confirmed whether the pH played any role in the layer formation in this study.

The macro-layers have been reported by Chao et al. (2000) in agitated-bioreactor (air-lift reactor). They observed the formation of two layers on the pellets with an increase in O₂ supply by 50%. A subsequent increase in the rate of BC production was also reported. These layers were not observed when only air was supplied to the agitated-bioreactor. The pH was not controlled in the bioreactor during air-supply and O₂ supply and similar growth conditions were maintained. Hence in the study of Chao et al. (2000) the layer formation could be attributed to the increase in the level of O₂. Similar impact of O₂ on the macro-layers has also been reported by Bodin et al. (2007a) in the BC tubes produced using the static-culture. They observed a decrease in the thickness of macro-layers with the decrease in supply of O₂. Their observation supports the role of oxygen in the control of the thickness of the macro-layers. Further investigations need to be carried to confirm the same in rotating-bioreactor. The pH was not controlled in the bioreactor hence the layer formation has been attributed to the increase in the level of O₂ concentration controls only the thickness of the macro-layers or their formation as well. In the present study, a constant supply of air was maintained in the rotating-bioreactor but the amount of dissolved oxygen in the medium was not monitored. It is therefore difficult to confirm whether there was a change in the O₂ level during the course of BC production. Additionally, it has been reported that the O₂ uptake of the bacteria changes with the change in pH and is maximum at pH 4 (Embuscado et al. 1994; Masaoka et al. 1993; Verschuren et al. 2000). If this hypothesis holds true then macro-layers should be observed in the BC produced at pH 4 in the present study but they were observed only when the pH was not controlled. The impact of O₂ on the BC morphology at different pH levels needs to be further investigated with the rotating-bioreactor.

3.6 Micro-layers in the BC structure

Micro-layers can be defined as layers observed in BC under the microscope usually in freeze-dried and air-dried samples (Fig.3.8-a). Micro-layers of approximately 200 μm were observed by our group in the freeze dried samples (Fig. 3.8-b) in both samples from static-culture and rotating-bioreactor methods (Schrecker 2004) in the past and also in the current study. Micro-layers were also observed in air-dried samples of BC produced in all the fermentation runs at varying growth conditions.

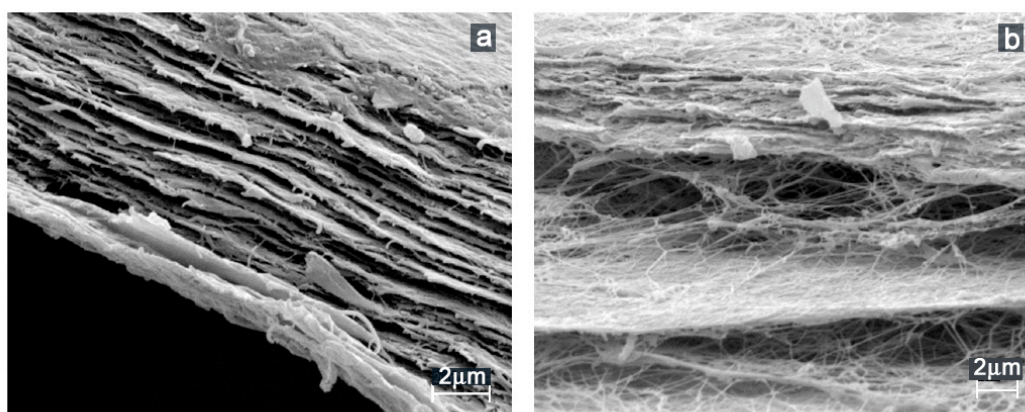


Fig. 3.8 - SEM micrographs of micro-layers observed in air-dried samples of BC produced in a rotating-bioreactor (a) and freeze-dried sample of BC produced in the static-culture (b) (Schrecker 2004).

3.6.1 Discussion

There are no published reports in literature analyzing the structure of BC produced in a rotating-bioreactor. Although there are reports on the structural differences of BC produced in an agitated-bioreactor and static-culture (Jonas and Farah 1998). The SEM micrographs published by Czaja et al. (2004) of wet BC produced in both static-culture (thicker fibrils and lesser inter-space between ribbons) and the agitated-bioreactor (finer fibrils with greater inter-space between ribbons) appear similar to the micrographs of

static-culture and the rotating-bioreactor respectively in the present study. The micro-layers were observed by Serafica (1997) in BC produced in a rotating-bioreactor and were referred to as lamellar layers. Micro-layers were also observed in the past by Yamanaka et al. (1989) but only in air dried BC samples produced in a static-culture. They suggested that vibrations in the BC culture vessel were the cause for these micro-layers though it was not confirmed. In yet another report, Yamanaka and Watanabe (1994) observed these micro-layers in dried, heat pressed BC samples produced in a static-culture. They referred to it as laminar layers and attributed their formation to the process of horizontal heat pressing. Schrecker (2004) demonstrated the formation of these layers was an artifact of the drying process, free from the influence of the gravitational force during production on the direction of layer formation. A similar observation during the de-sorption process, in the pellicles produced by the static-culture was made by Gelin et al. (2007) although they reported a formation of BC-pleats and not complete layers.

3.7 Influence of pH on the width of BC ribbon

3.7.1 BC ribbons produced in a static-culture

The BC pellicles (by any method of production) are produced by the aggregation of the BC ribbons that are formed from the microfibrils at the cell surface (Brown et al. 1976). Thus, the width of the BC ribbon has a direct impact on the pellicle formation that is quantified as the final yield. There have been no reports in the past on the impact of varying pH on the morphology of the BC in the static culture hence it was investigated. BC was produced in the static-culture by inoculating cultures in the medium at different initial pH values (2.4, 4, 5, 6 and 7) in triplicate. It was difficult to control the pH during BC production in a static-culture; hence the pH was adjusted only at the time of inoculation using 0.5 N, acetic acid and left undisturbed for a period of 10 days. A BC membrane appeared in all the cultures on the third day. Similar growth medium with

glucose concentration of 50 g/L was used and equal volume (1 ml) of inoculum was added to each culture. The medium samples were taken for glucose analysis only on the day of inoculation and on the day of harvest. Sufficient BC was not produced at an initial pH of 7; therefore it was not used for SEM analysis. After harvest, the samples were freeze-dried and observed under SEM and their respective ribbon width was measured for comparative analysis. The width of individual BC ribbons was measured at the widest part (chapter 2, section 2.26.7) (Yamanaka et al. 2000). There was no significant difference in the ribbon width and it was approximately 94 ± 39 nm (standard deviation) for all the samples observed (sample size = 30 for each pH). The only difference that was noticed was excessive scarring in the BC samples produced at pH 2.4 compared to the samples produced at pH 4, 5 and 6 (Fig. 3.9).

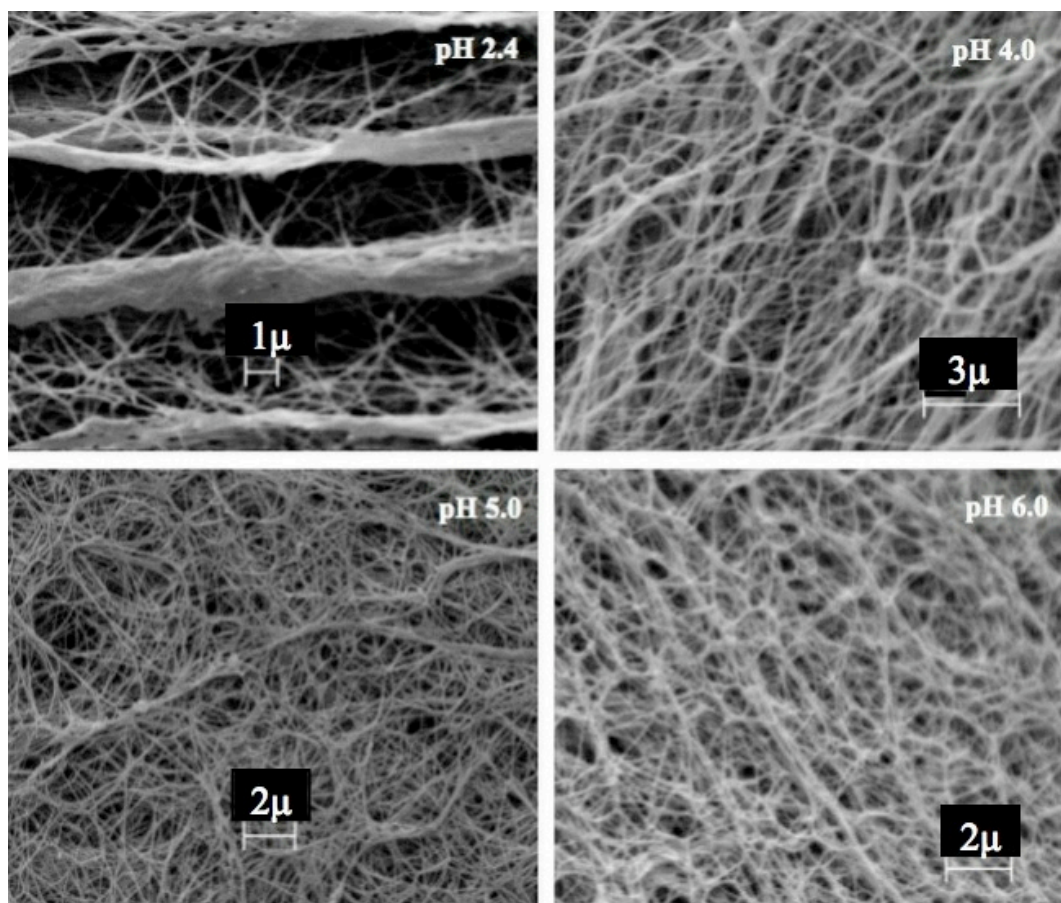


Fig. 3.9 – The representative SEM micrographs of the freeze-dried samples of BC produced in a static-culture at different initial pH of 2.4, 4.0, 5.0 and 6.0 as labelled in the figure.

3.7.2. BC ribbons produced in a rotating-bioreactor

In order to investigate the impact of pH on the micro-structure, BC was produced using the rotating-bioreactor at pH values from 2.0 to 6.0. At pH 2.0, no BC was produced and when the pH was gradually raised to pH 2.45 using caustic, the BC film appeared but was not enough to form a pellicle. A pellicle of thickness sufficient for post-production analysis was produced only at pH 3.0 and above. The SEM micrographs of BC samples produced in a rotating-bioreactor at different pH and without pH control were analyzed to determine ribbon width (Fig. 3.10) using Image-Pro and compared with the micrographs of BC produced in the static-culture (Fig. 3.11).

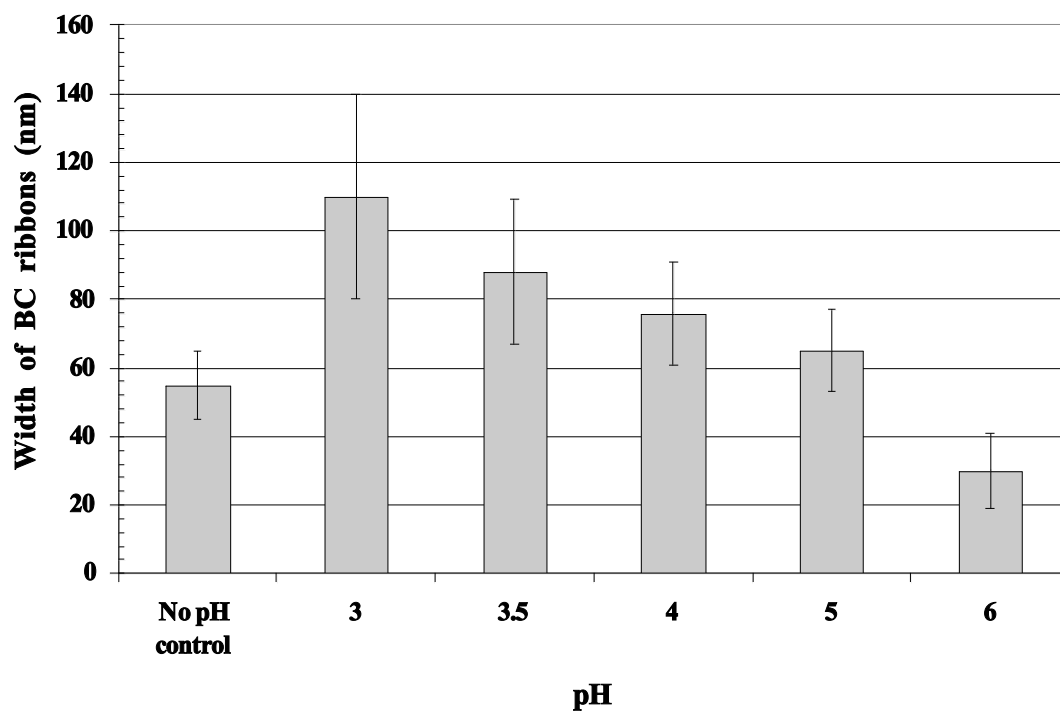


Fig. 3.10 –The average ribbon width of samples produced in a rotating-bioreactor controlled at different pH (3-6). For the samples (no pH control) large sheet-like areas were present that were not seen in other samples and the relatively fewer individual ribbons were observed. The variability represents the difference between the width of the ribbons measured in different samples of BC (sample size = 30).

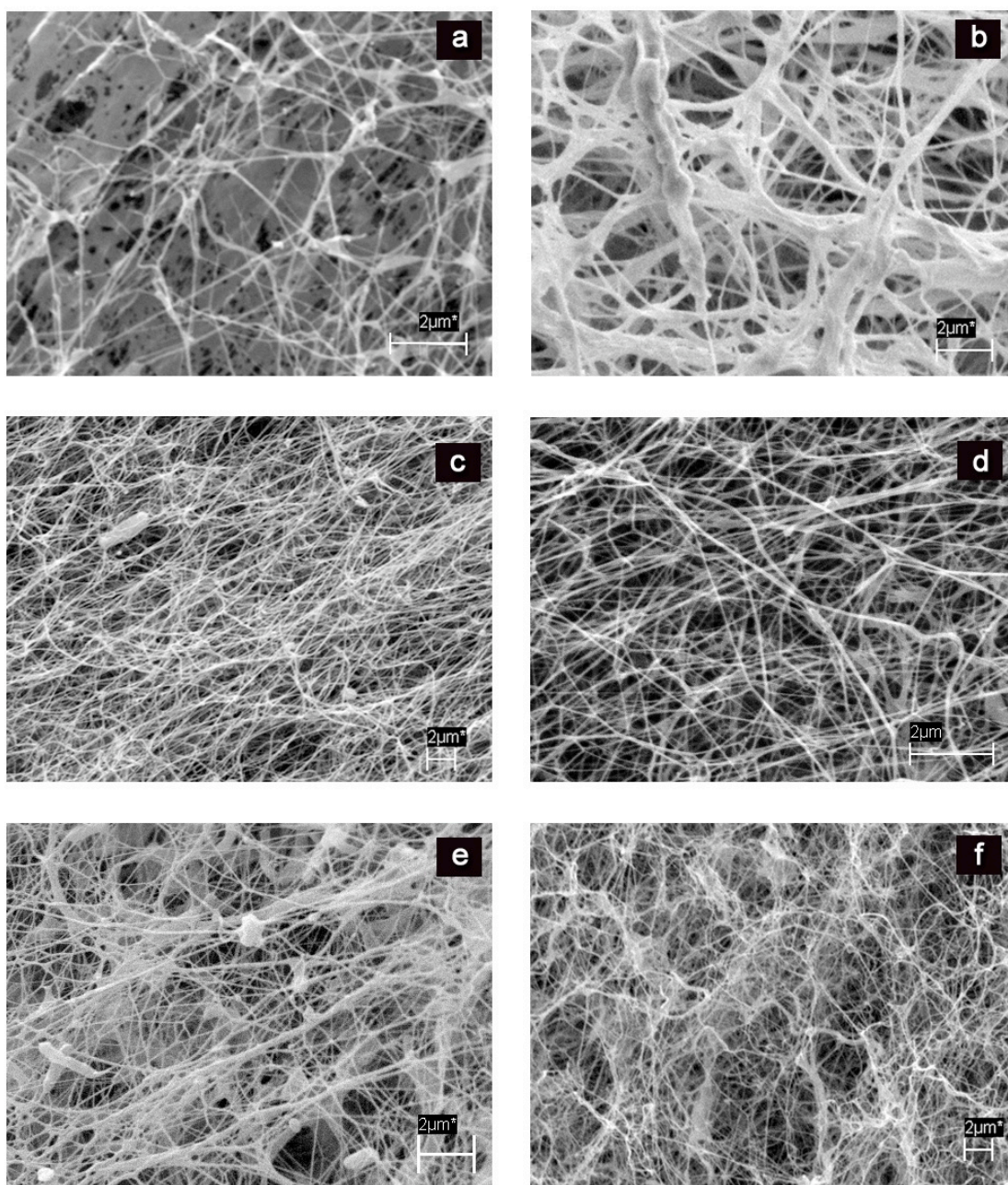


Fig. 3.11 – The SEM micrographs of the freeze-dried BC produced in a rotating-bioreactor at different pH. a – BC produced without pH control; b – pH 3.0; c – 3.5; d – pH 4.0; e – pH 5.0; f – pH 6.0.

3.7.2.1 Discussion

The results suggest that there was a reduction in the width of the ribbon with the increase in the pH (Fig. 3.11). Statistical analysis (ANOVA) showed a significant difference between the widths of the ribbons produced at different pH ($p < 0.05$ at 95 % C.I). The observations and statistical analysis suggest that the ribbon width was greater at pH 3.0 (110 ± 30 nm) than at pH 6.0 (30 ± 8 nm). Similar reductions in ribbon width are reported in another study at high pressure (Kato et al. 2007). They hypothesize that possible compression of the lipopolysaccharide membrane (outer cell membrane) impacts the pore size and/or number that could result in finer fibrils. In the current study, the change in pH could have affected the conformation of the pores causing them to produce finer ribbons at higher pH, although this hypothesis needs to be confirmed with further investigations.

While the reasons for the difference in width of the BC ribbons at different pH are not clear, there have been some assumptions in the past for thicker than regular ribbons. The interwoven BC network is hypothesised to be the result of cell division (Watanabe and Yamanaka 1995; Yamanaka et al. 2000; Yamanaka et al. 1989). At each cell division, every individual cellulose ribbon formed divides longitudinally into two. This happens repeatedly with each cell division, leading to the formation of the observed BC network (Yamanaka et al. 2000). In the absence of regular cell division, the ribbons start joining into thicker ribbons instead of splitting into two. This has been shown in the presence of antibiotics that reduced the cell division rate, leading to the formation of thicker BC ribbons although the exact measurement was not reported (Watanabe and Yamanaka 1995; Yamanaka et al. 2000; Yamanaka et al. 1989). There have been reports of thicker BC ribbons when produced in the presence of terpenoid (Haigh et al. 1973) although the exact reason for the same was not elucidated. There could be a possibility that at the lower pH the cell division is slower leading to thicker ribbons. Although this has not been

established directly, there have been reports that the maximum rate of production and yield of BC is observed between pH 4.0-5.0 (Embuscado et al. 1994; Hestrin et al. 1947; Lapuz et al. 1967; Masaoka et al. 1993; Toda et al. 1997; Verschuren et al. 2000) and BC production is directly related to cell division thereby an indirect relationship between pH and ribbon thickness can be established. This is just a hypothesis at this stage and would require further investigation to confirm the same.

Another theory suggests that certain additives, such as calcium fluoride encroach on the hydrogen binding sites interfering with the regular ribbon formation (Astley et al. 2001). While still other research suggests that the interference in the movement of the cells which is important for the crystallization of cellulose could impact the ribbon formation. Since the rotation of the cell around its longitudinal axis is responsible for the ribbon assembly from the nascent fibril (Hirai et al. 1997). In another study, Brown (1976) reported that the BC ribbons are formed due to the close and well synchronised extrusion of glucan chains from various pore sites. They also confirmed that approximately 46 microfibrils combine to form a single ribbon and that if the extrusion of these glucan chains is affected in any way, the ribbon formation gets impacted. If the pH can impact the cellulose extrusion it can directly influence the dimensions of the ribbon.

Throughout the literature, various researchers have pursued different techniques for observations of BC ribbons and their subsequent measurement. In the present study, the freeze-dried samples of BC from the runs at different pH were observed using SEM since this technique maintains the structure without obscuring the pores (Klemm et al. 2001). The BC ribbons were an entangled mesh hence it was very difficult to measure the length of individual ribbons. There was also no simple way to determine the number of microfibrils that made up the measured individual BC ribbon. In the past the width of the

microfibrils has been estimated as approximately 3-4 nm (Brown et al. 1976;Haigler et al. 1982;White and Brown 1981). This has been achieved by observations *in vivo* with the help of darkfield light microscopy (Brown et al. 1976) and *in vitro* by SEM (Haigler et al. 1982). In the current study, the microfibril width was not measured, only the ribbon-width was calculated based on SEM micrographs using the ImagePro software (Chapter 2, section 2.16.7). This ribbon-width estimation method has been used with SEM and AFM in several studies (Astley et al. 2001;Brown et al. 1976;Hirai et al. 2004;Klemm et al. 2005;Yamanaka et al. 2000). In the early reports, Wyssling and Muhlethaler (1946) reported that all the cellulose ribbons were of equal diameter and 20 nm thick based on the assumption that the ribbons were cylindrical in shape. Kaushal et al. (1951) reported that the ribbon width varied between 20-25 nm and established that they were rectangular in shape. In more recent studies, the estimated width of the BC ribbon produced under static culture was reported to be 133 nm (Brown et al. 1976) determined with the help of darkfield light microscopy, Astley et al. (2001) reported it as approximately 50 nm using an environmental SEM. The width of the BC ribbon as determined by Haigler et al. (1982) using SEM was between 40-60 nm and Tokoh et al. (1998) was between 30-50 nm as observed under transmission electron microscopy (TEM). The variation in the reported ribbon-width could be a result of the difference in techniques used by the different research groups.

There are published studies on the different methods of measurement of the microfibrils and ribbon width of the BC produced in static-culture in addition to the reports on the production of BC, at different pH in static-culture (Yuhong et al. 2006). However, there are no published reports on the comparative study of micro-structural features including ribbon width as reported in the current study. There was a report of BC production in a rotating-bioreactor at different pH (Kim et al. 2007). However, this study was more

focused on the bioprocessing aspect and did not mention about morphology of the BC produced.

The BC microfibrils maintain their structure within the pellicle due to the adsorbed water molecules between them (Colvin and Leppard 1977). The ribbons are formed by the aggregation of these microfibrils due to Van der Waals forces of attraction on removal of adsorbed water (Colvin et al. 1977). The amount of water released by the microfibrils could vary during the freeze-drying process, which could contribute to the variation in ribbon width in a given sample. An artifact if any, of the freeze-drying process can be confirmed by observing the BC samples in an ESEM in the wet (never-dried) state.

In an unrelated study of another Gram-negative bacteria, *Vibrio cholerae*, it was observed that the bacteria was able to alter its structure and physiology even with a moderate decrease in pH (Hommais et al. 2002). The reduced pH consequently led to the fall in production of the pH-regulated proteins including different proteins involved in the formation of membranes. This typical stress response to change in environmental conditions, including lowered pH, involves modifications of the membrane proteins (Kadner et al. 1996). This adaptation has also been observed in other bacteria including *E. coli* and *Salmonella* (Kadner et al. 1996). Since such a stress response is common in most bacteria, it is quite possible that *G. xylinus* responds in a similar manner to a change in pH. If the hypothesis holds true, then the crystallization of BC could be impacted because the pores positioned on the outer membrane also function as sites enabling the crystallization of glucans into microfibrils (Zaar 1979). Analysis of the amount of different proteins involved in the membrane formation should be compared within cells produced at different pH in order to confirm the same.

3.8 Control

As a control, some harvested BC samples from the rotating-bioreactor produced at pH 4.0 were immersed for 10 days in glucose medium at different levels of pH 2.0, 3.0, 4.0 and 5.0. The pH was adjusted using, 0.5 N, acetic acid. Even at pH 2.0, there was no observed change in structure (Fig. 3.12). Macro-layers were not observed at any pH level indicating that if pH influenced the structure then it did so during the BC formation stage and cannot be influenced post-harvest. The ribbon-width too was unchanged in control samples. The influence of pH on the BC structure should be a subject of further study.

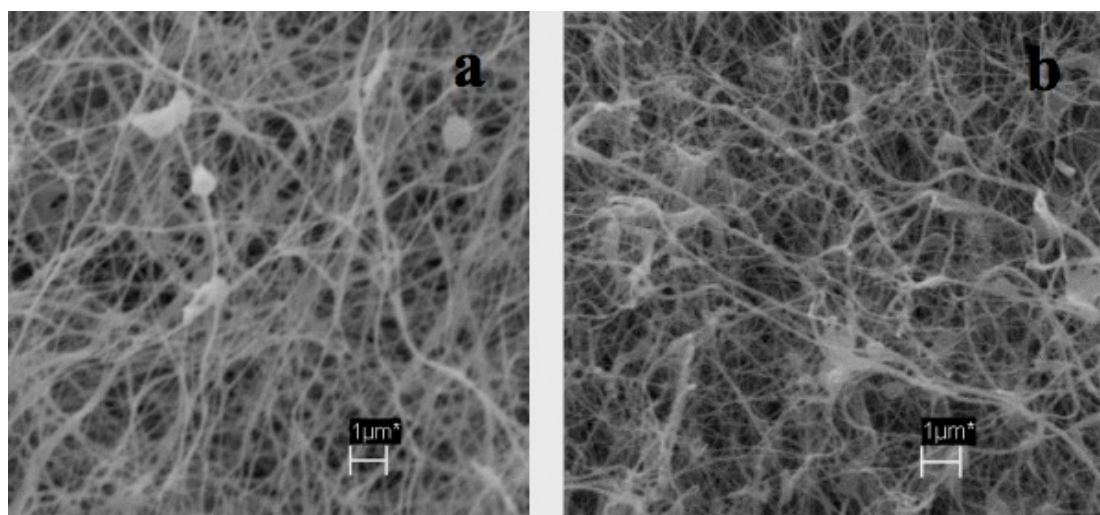


Fig. 3.12 –The SEM micrograph of a freeze-dried sample of BC produced in a rotating-bioreactor at pH 4.0 (Fig. 3.12-a) and micrograph of a freeze-dried sample of the same sample immersed for 10 days in glucose medium at pH 2 using 0.5N, acetic acid (Fig. 3.12-b).

3.9 Influence of change in pH on the BC morphology produced in RBC

Experiments were performed to investigate the changes in the structure when the pH was changed in a controlled fashion during the BC production in a rotating-bioreactor. The first run was controlled at pH 3.0 for 72 hours following lag phase (48 hours) and at pH 4.0 for 72 hours. In the second run, the conditions were reversed; the rotating-bioreactor was controlled at pH 4.0 for 72 hours followed by pH 3.0 for 72 hours. Macro-layers were not observed in the pellicles formed in either sample but once the samples were freeze-dried and observed under SEM some interesting observations were made.

Micro-layers were observed perpendicular to the direction of pellicle formation (Fig. 3.12-a) when the system was controlled at the pH 3.0 at the beginning of the run. These micro-layers (thickness = 2-4 μm) were closely packed (Fig. 3.12-a). The overall BC structure was similar to that in runs produced without pH control (Fig. 3.11 b) and in BC produced in a static-culture (Fig. 3.7-b). When the pH was raised from 3.0 to 4.0 after 72 hours, there was a structural change in the micro-layers. The micro-layers formed under pH 4.0 were thicker (thickness=60-130 μm) and more loosely arranged (Fig. 3.12-a). Scarring was observed in almost equal areas in both parts of the pellicle produced in pH 3.0 and 4.0.

The conditions of pH control were reversed in the next fermentation run. The run was controlled at pH 4.0 during the first half of the total duration of the run (72 hours) excluding lag phase (48 hours) and then reduced to pH 3.0 during the second half (72 hours until harvest). Few and fine micro-layers were observed in the first half of the duration of pellicle formation in pH 4.0 (Fig. 3.13-b). When the pH was reduced to 3.0 in the second half of the fermentation, micro-layers were observed (thickness = 60-120 μm) (Fig. 3.13-b). These micro-layers were similar to those observed at pH 4.0 in the previous

run (Fig. 3.13-a). Additionally, there was a difference in the orientation of these micro-layers as they were arranged parallel to the direction of pellicle formation (Fig. 3.13-b). Limited scarring was observed in parts of the pellicle produced at pH 4 in comparison to the parts of pellicle produced at pH 3 (Fig. 3.13-b). The structure produced at pH 4.0 resembled the structure observed in BC produced in the rotating-bioreactor at a controlled pH of 4.0 throughout the run (Fig. 3.11-d). While the structure produced at pH 3.0 resembled the structure produced at pH 4.0 in the previous run barring the orientation of the micro-layers (Fig. 3.13-a and 3.13-b).

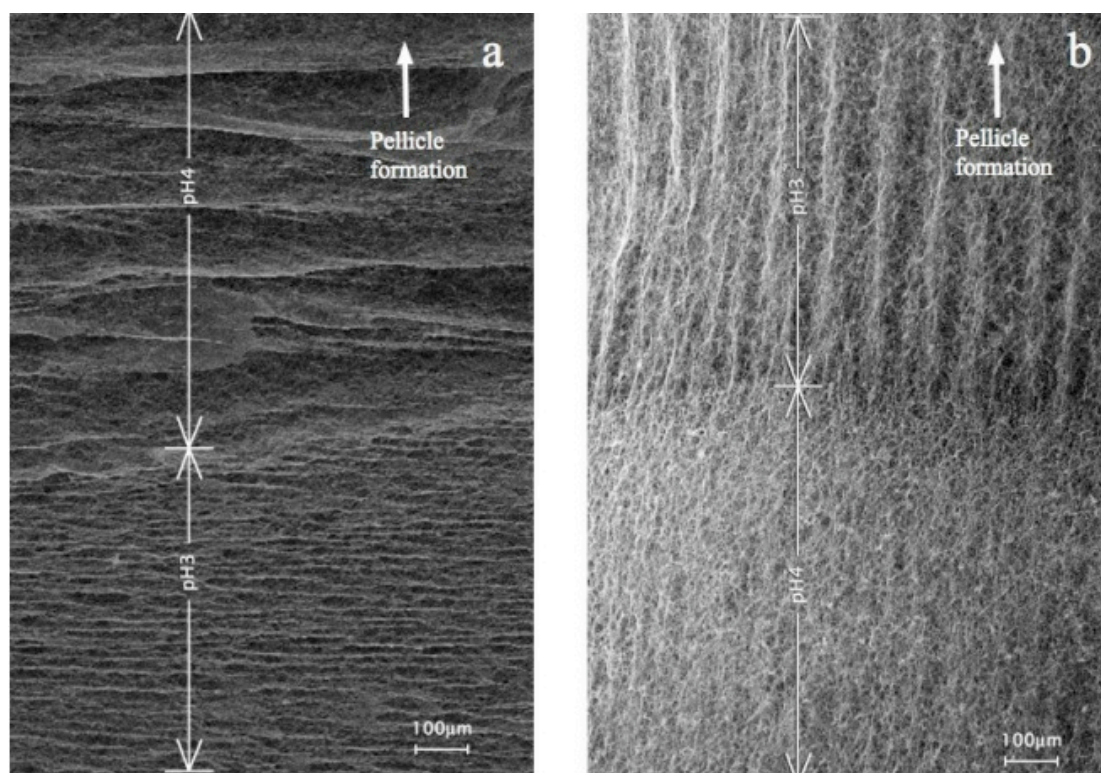


Fig. 3.13 - In (3.13-a), the rotating-bioreactor was controlled at pH 3.0 for 72 hours and at pH 4.0 for 72 hours. In (3.13-b), the pH control was reversed and was controlled at pH 4.0 for 72 hours followed by pH 3.0 for 72.

3.9.1 Discussion

While a difference in the morphological structure has been observed at different pH values and shifting the pH during production, the reason for the same is unclear. The assumption is that the difference in pH is the causal factor for the apparent change in the BC ribbon morphology because most other parameters of growth and medium conditions were controlled. The only major exception was falling glucose level in the medium with the simultaneous rise in gluconic and acetic acid level. Further studies are required to investigate whether the increasing acid level and the decreasing glucose levels have any influence on the BC structure. Although, as per published report the degree of cellulose polymerisation was not impacted by the variation in the pH of the growth medium due to increased acid production (Marx-Figini and Pion 1974).

The only published study that supports the hypothesis that the pH impacts the morphology of the BC was by Ben-Hayyim and Ohad (1965). They used carboxymethylcellulose (CMC) to reduce the electro-static charge in the fibrils increasing their conglomeration. They also reported that the pH reduction reduced the electro-static charge of the cellulose fibrils. While the current study did not use CMC, there was a tendency to observe thicker cellulose ribbons and greater degree of aggregation in the BC samples (as observed under SEM) in lower pH of 3.0 and 3.5 in contrast to that observed at a higher pH of 5.0 and 6.0 (Fig. 3.11). Additionally, it would provide a plausible explanation as to why the structure changed from a closely knit or a tighter structure, to a looser structure when the pH was reduced from 4.0 to 3.0 (Fig. 13.3-b). A similar reversed effect was observed when the closely knit structure at pH 3.0 changed into a loosely knit structure when the pH was changed to 4.0 due to increased formation of finer ribbons (Fig.13.13-a). Another theory suggests a change in structure when the pH was reduced from 4.0 to 3.0 could possibly be due reduction in biofilm formation as observed

in the bacteria *Vibrio cholerae* (Hommais et al. 2002). When the pH was reduced from 4.0 to 3.0 the reduction in BC ribbon formation could have made the pellicle with more compact macro-layers. The reversal of the pH of the medium from 3.0 to 4.0 may have increased BC production and led to the ribbons forming a more open structure. This theory needs further investigation.

It has been reported that a pH change beyond its optimal range adversely affects the cell membrane of most micro organisms by impacting its structure and permeability (Sinclair 1987). A structural change in the membrane of *G. xylinus* could impact either the function and/or the number of terminal complexes (TC) and the pores present in the membrane that direct the extrusion and crystallization of BC ribbons (Brown et al. 1976; Hirai et al. 2004; Zaar 1979). Additionally, as mentioned earlier there are published reports that suggest reduced production of protein required for cell membrane formation (Hommais et al. 2002) that could impact the cellulose extrusion from the membrane based pores. This change in pH could impact the BC ribbon formation and affect the structure of the pellicle but whether these structural changes in the cell membrane brought about by the change in pH are permanent or reversible remains to be determined to confirm this as a possible factor.

The structural difference observed in the Fig. 3.13 could be due to the change in structure influenced by the variation in pH as mentioned in the earlier sections. It was previously established that the orientation of the BC layers were not influenced by gravity when air-dried (Schrecker 2004). Although this kind of structural variation was not observed in any of the runs with constant pH or naturally dropping pH, more runs are needed with greater variation in pH to confirm the observations. The bacterial cells should be harvested at different pH values and the various membrane forming proteins should be extracted and

evaluated in order to ascertain whether there is reduction in the same due to stress response. Additionally, BC samples should be taken before and after the pH change for a better comparative analysis. If possible, the samples should be observed under an environmental SEM where the structure can be observed in the hydrated form without freeze-drying.

3.10 Size and distribution of bacterial cells in BC

Marx-Figini and Pion (1974) proposed that the BC production is directly related to the number of viable cells. In order to find the bulk average of cells in the BC, different methods have been used by various researchers, including plate count technique (DeWulf et al. 1996;Ishida et al. 2003;Kato et al. 2007;Marx-Figini and Pion 1974), dry weight of cells (Ben-Hayyim and Ohad 1965;Joseph et al. 2003;Krystynowicz et al. 2000) and optical density (Chao et al. 2000;Hwang et al. 1999;Kim et al. 2007;Kouda et al. 1997b). In the current study, the number of cells and their distribution within the matrix of the BC ribbons were investigated using confocal laser scanning microscopy. SEM was not used as it was difficult to differentiate the bacteria from the cellulose as they were both white in colour. Besides, only a two-dimensional view of the bacterial cells and their distribution could be observed.

Confocal scanning laser microscopy (CSLM) can observe live cells and their three dimensional distribution within the BC network. The BC samples from both the static-culture and the rotating-bioreactor were not treated with caustic after harvest in order to retain the bacterial cells. The viable cells were stained with rhodamine blue dye (López-Amorós et al. 1997). Cells were readily visible upto a depth of 120 μm . The ImagePro software was used to count the number of cells and their distribution (Chapter 2, Section 2.16.7). The observations of the cells are expressed per unit area because there the image

slices were very fine (0.6 μm) this resulted in the overlapping of the cells (in part or whole) in different planes. Thus the cells could not be measured accurately per unit volume. The BC samples from the rotating-bioreactor were produced under following conditions (initial glucose concentration = 50 g/L at pH 4, tangential velocity = 0.095 m/s and submersion level: $C_L = 26\%$ and $C_S = 21\%$). The BC samples (no pH control) was produced under similar conditions but without active pH control. The static-culture sample was produced at an initial glucose concentration of 50 g/L and initial pH of 4.5.

3.10.1 Observation of live cells in BC

The number of cells observed under CSLM was estimated from the images taken using the ImagePro software (Table 2). The cell density was greater in the static-culture samples in the upper layer as compared to the rotating-bioreactor samples (Table 2). Additionally, the bacteria in the static-culture samples appeared to be closely associated with one another in the form of long chains and many such chains were observed and fewer bacteria appeared in isolation (Fig. 3.14-a and 3.14-b). Based on the dimensions of an individual bacteria 0.6-0.8 x 1.0-4.0 μm (Holt et al. 1994), it was evaluated that approximately 8 ± 3 bacteria per chain were associated in the upper layer of BC from the static-culture (Fig. 3.14-a). Greater numbers of cells were observed in the upper layer, 892 ± 54 , but fewer cells, 25 ± 10 , in the bottom layer albeit in longer chains (11 ± 4) (Fig. 3.14-b) (Table 2). In contrast, the bacterial cells in the upper layer of the BC produced in the rotating-bioreactor without pH control were fewer in number 289 ± 31 (Table 2) in comparison with the static-culture. They were scattered around either as individual or as very few short chains of not more than 2 ± 3 bacteria per chain (Fig. 3.15-a). But there were more bacterial cells 106 ± 20 in shorter chains 1 ± 2 (Fig. 3.15-b) in the bottom layer of the rotating-bioreactor cellulose as compared to the BC samples produced in static-culture. The least number of cells/ mm^2 were observed in the uppermost

layer of BC produced in the rotating-bioreactor at pH 4 (2560 ± 27) as compared to no pH control (4624 ± 31) and in the static-culture (14272 ± 54) (Table 2). The cells were widely distributed and observed in very short chains (1 ± 2) although, seldom (Fig. 3.16-a). The cells/mm² in the bottom layer of BC produced without pH control (1696 ± 18) was marginally greater than that observed at pH 4 (1376 ± 20) but greater than that observed in lower layer of BC produced in static-culture (400 ± 10) (Table 2). The error analysis of number of bacterial cells estimated for all the samples is mean \pm standard deviation and samples size =50 for each treatment.

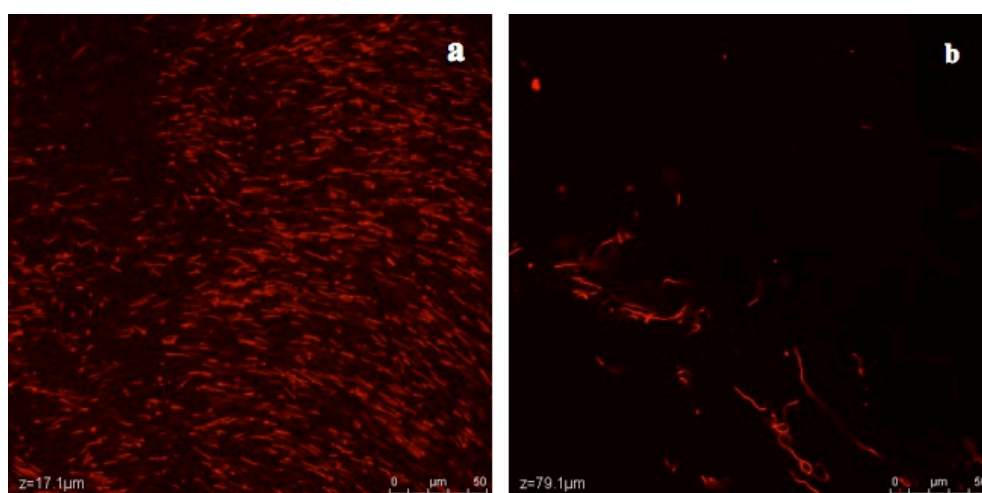


Fig. 3.14 - A representative CSLM micrograph of the uppermost (a) and the bottom (b) layer of the BC sample(area = 0.0625 mm²) produced in a static-culture.

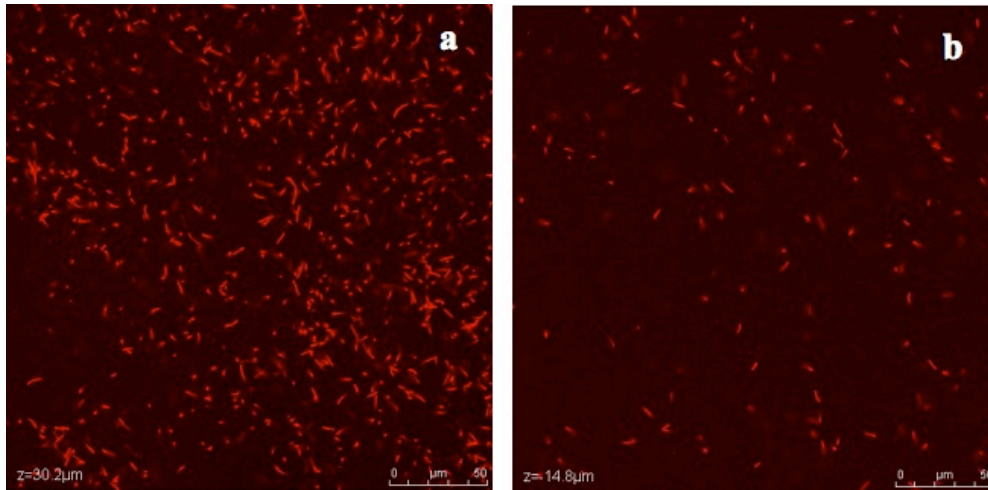


Fig. 3.15 - A representative CSLM micrograph of the uppermost (a) and the bottom (b) layer of the BC sample (area = 0.0625 mm²) produced in a rotating-bioreactor without pH control.

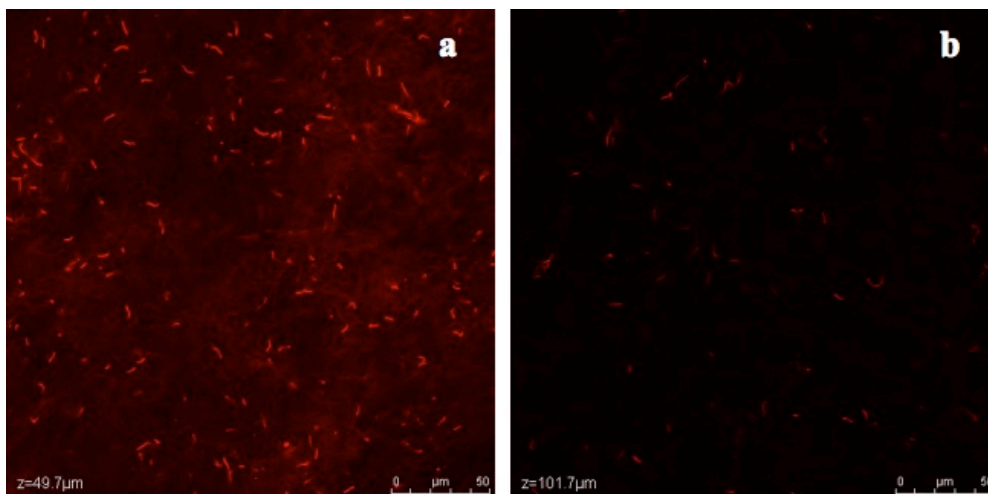


Fig. 3.16 - A representative CSLM micrograph of the uppermost (a) and the bottom (b) layer of the BC sample (area = 0.06225 mm²) produced in a rotating-bioreactor controlled at pH 4.0.

The number of cells in the uppermost layer of the BC samples from the static-culture was three times greater than those observed in the BC samples taken from a rotating-

bioreactor. The ratio of the number of cells at the upper surface to the bottom of BC in the static-culture was approximately 16:1 in comparison with the rotating-bioreactor which was 3:1. Whereas, the number of cells on the upper most surface of the BC produced without pH control showed the presence of greater number of cells in comparison to the BC produced in pH 4.0 but lesser than those found in the static-culture.

Table- 2 - The average number of cells observed in five different locations on the upper and bottom layers. The observations were recorded at a depth of 100 μm from the top and bottom of the pellicle of each of the BC samples (sample size = 10). The area of observation was 0.0625 mm^2 in each sample produced in both the static-culture and in the rotating-bioreactor at pH 4 and without pH control respectively. The error analysis is mean \pm standard deviation.

Observed area	Static-culture (average # of cells/ mm^2)	Rotating- bioreactor (pH4) (average # of cells/mm^2)	Rotating- bioreactor (no pH control) (average # of cells/mm^2)
Uppermost layer	14,272 \pm 54	2,560 \pm 27	4,624 \pm 31
Bottom layer	400 \pm 10	1,376 \pm 18	1,696 \pm 20

3.10.2 Discussion

Rhodamine blue only stains viable cells but it is difficult to conclude whether all the cells stained are cellulose producing cells or non-cellulose producing mutants. This information is important in BC produced in a rotating-bioreactor as the chances of non-cellulose producing mutants are higher due to increased agitation (Krystynowicz et al. 2002; Verschuren et al. 2000). In the current study, the difference between the cellulose producing and non-producing cells was not established. However, it was assumed that all the stained, viable cells were cellulose-producing to arrive at certain conclusions. Larger numbers of cells were widely distributed in the BC produced in the static-culture compared to the rotating-bioreactor. This observation in the present study are supported by the reports of Kinsey et al. (2005) who made similar observation of lower cell density in the rotating-bioreactor as compared to the static-culture although the reason for the same was not elucidated. The possible reason could be that in the static-culture after the initial pellicle formation, most of the cells get concentrated in the upper layer of the pellicle. This observation also explains to some extent why the fibrils are closely knitted in the static-culture (Fig.14-b) as compared to a rotating-bioreactor because of smaller difference in the proximal distance of the cellulose producing cells. In the rotating-bioreactor due to the constant agitation of medium by rotation of the cylinders, the cells were dispersed in the medium. Thus fewer cells are available to form the pellicle on the cylinders. This offers an explanation for the more open morphological structure in BC produced in the rotating-bioreactor (Fig. 3.3-d). It also offers an explanation for the formation of BC on the non-cylinder areas of the reactor (referred to as slop). Although, the reason for more cells in BC produced without pH control than when controlled at pH 4.0 in a rotating-bioreactor is not clear, absence of environmental stress could be one of them (Hommais et al. 2002). The reason for a closer knit cellulose structure can be

explained by the presence of greater number of cells in the upper layer of pellicles produced without pH control in comparison with the BC produced at pH 4.0 in the rotating-bioreactor.

More cells per unit area are observed in the uppermost layer of samples produced in both the static-culture and the rotating-bioreactor as compared to their respective lowermost layers. This could be because of higher accessibility of substrates specially oxygen in the BC pellicles produced in both the rotating-bioreactor and the static-culture (Serafica 1997). The lesser number of cells observed in the lower layer in the static-culture compared to the rotating-bioreactor produced at pH 4.0 could be due to higher cells motility in static-culture as a direct impact of lower diffusivity of oxygen. Alternately, the cells could migrate to the upper surface of the growing pellicle. The number of dead cells at the bottom most layer should be determined in order to confirm the same. The cells in the BC pellicles produced in the rotating-bioreactor are alternately exposed to air and medium hence have greater access to the substrates. Despite this fact there are fewer cells in the lower layers of BC produced in the rotating-bioreactor this could be because the average depth of oxygen diffusion was approximately 875 microns before it was used up by the cells (Serafica 1997). The average thickness of the pellicle was 5 cm hence insufficient oxygen could have resulted in cell death at lower layers leaving fewer viable cells.

The reason for formation of chains of cells observed in the upper layer of BC produced in static-culture cannot be explained but the higher number of cells and their consequential proximity could be the possible reason for the same. In contrast the probable reason for shorter chains in the rotating-bioreactor could be due fewer numbers of cells subjected to constant agitation of the medium by the rotating cylinders. But the presence of more chains and cells in BC produced in the rotating-bioreactor without pH control compared

to that produced at pH 4 cannot be explained and would require further investigation. Assuming all conditions of growth to be similar including RPM or tangential velocity in the rotating-bioreactor, the only difference was the pH, but if pH was the only reason or only one of the contributing reasons needs to be investigated. While the reason for the formation of chains by the bacterial cells is elusive, it offers an explanation for the difference in the morphological structure observed in (Fig. 3.13-a) and (Fig. 3.13-b). The uniplaner movement of a large number of bacterial cells associated in chains parallel to the direction of the growing pellicle (Putra et al. 2008a) may possibly cause overlapping of the ribbons produced. This could offer an explanation for the formation of macro-layers observed in BC pellicles produced in static-culture but there is still no explanation for the presence of macro-layers in the rotating-bioreactor formed only without pH control.

Although there are reports in the literature on using CSLM (Chanliaud and Gidley 1999), there have been no published reports on the size and distribution of bacterial cells in either the static-culture or the rotating-bioreactor produced BC. Few samples produced in static-culture and the rotating-bioreactor was observed and CSLM observations were not carried out for most runs as the majority of samples were boiled in caustic to remove the bacteria. In future, CSLM observations should be conducted for the BC harvested from a rotating-bioreactor controlled in different growth conditions with respect to pH and tangential velocity for further investigations.

3.11 Comparative analysis of BC produced in both a static-culture and a rotating-bioreactor using X-ray diffraction

In the past various structural studies were conducted on cellulose using electron-diffraction analysis (Sugiyama et al. 1991) and Nuclear Magnetic Resonance (NMR)

(Atalla and Vanderhart 1984; Yamamoto et al. 1996) and X-ray diffraction (Czaja et al. 2004; Hirai et al. 1997; Klemm et al. 2005; Mondal and Kai 1999; Yano et al. 2008). These studies confirmed the presence of cellulose in two different crystalline phases I α and I β . It has been observed that cellulose obtained from *G. xylinus* is usually made up of highly crystalline I α -cellulose (Atalla and Vanderhart 1984; Czaja et al. 2004). In the present study the effect of the different method of production: rotating-bioreactor and static-culture on the crystalline nature of the BC produced was investigated.

Samples of dry BC produced in a static-culture (initial glucose concentration = 50 g/L) and in a rotating-bioreactor (initial glucose concentration = 50 g/L at pH = 4, tangential velocity = 0.095 m/s and submersion level: C_L=26% and C_S=21%) were analysed using X-ray diffraction. The monoclinic unit cell description by Sugiyama et al. (1991) was used to assign the peaks. The positions of the peaks and their respective widths were measured from plots by the method described by Gjønnnes and Norman (1958).

The diffraction angles were taken from the local maxima in the equatorial profiles of the diffractograms. Bragg's equation was used to calculate the interplanar spacing's, (d) where the main peak angle (θ) was substituted in the Eq.1.

Eq. 1. Bragg's equation:

$$d = \lambda / (2 \sin \theta) \quad (\text{Eq. 1})$$

where λ is the wavelength of the radiation.

The curves were then deconvoluted into Gaussian curves with a correlation coefficient greater than 0.98. In order to determine the crystallite dimension, the half-width at half-height of the peak assigned to 200 (planes) was calculated and used in the Scherrer equation:

$$T = K \lambda / (B \cos \theta) \quad (\text{Eq. 2})$$

where, T is the crystal thickness, λ is the radiation wavelength, θ is the diffraction angle and B the diffraction peak width measured at half maximum height (Davidson et al. 2004). The correction factor K usually set at 0.9 for this definition of B (Davidson et al. 2004). The crystallinity index CrI was determined as defined by Segal et al. (1959).

$$\text{CrI} = 100(I - I')/I \quad (\text{Eq. 3})$$

Where I is the height of the peak assigned to (200) planes, typically located in the range $2\theta = 21^\circ$ to 22° , and I' is the height measured at $2\theta = 18^\circ$, that is where the maximum appears in diffractograms of cellulose.

X-ray diffraction showed that both the BC samples were highly oriented (Fig. 3.16). The X-ray diffraction patterns of both the rotating-bioreactor and static-culture samples demonstrate typical cellulose I profile (Fig. 3.17). Additionally, the peaks assigned to (200) planes appeared at $2\theta = 23.08^\circ$ for the BC produced in static-culture and $2\theta = 22.86^\circ$ for the BC produced in rotating-bioreactor. These peaks were similar to the diffractograms published for cellulose I (Ishii et al. 2003; Isogai et al. 1989). The calculated peak widths for (200) planes using Bragg's Eq.1, full width at half maximum height were: static-culture = 1.66° , rotating-bioreactor = 1.45° . The Scherrer Eq. 2 was used to calculate the cross-sectional dimensions of the crystallites: static-culture $L(200) = 4.9 \text{ nm}$, rotating-bioreactor $L(200) = 5.6 \text{ nm}$. The crystallinity index was calculated using the Eq. 3 and it was estimated as 89.4% for the cellulose produced in the static-culture and 83.0% when produced in the rotating-bioreactor. The diffractogram of static-culture showed a peak assigned to the (004) planes (Fig. 3.17). This peak was absent in the diffractogram of BC produced in the rotating-bioreactor. The absence of this peak suggests a longitudinal disorder.

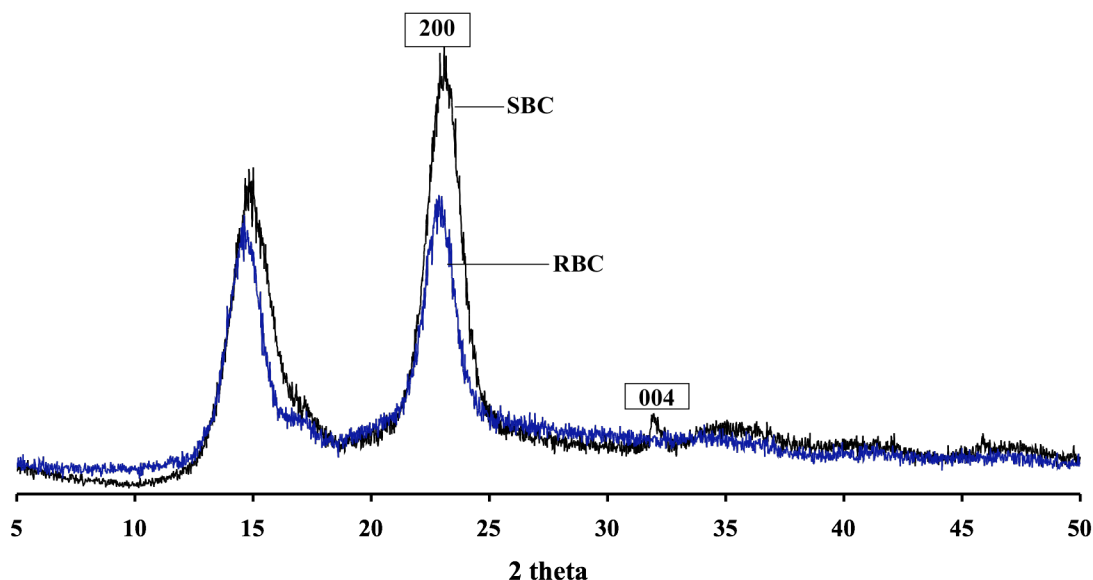


Fig. 3.17 - X-ray diffraction patterns obtained from representative BC samples produced in both the static-culture and in the rotating-bioreactor.

3.11.1. Discussion

The highly oriented samples observed in the diffractograms were indicative of rectangular (ribbon-like) nanofibres (Fig. 3.17). This observation was in agreement with the hypothesis of Kaushal et al. (1951) that the BC ribbons are rectangular in shape and not round. The cross-sectional dimensions of the microfibrils are within the range of 4-7 nm and are comparable to previous studies (Klemm et al. 2005). However, the difference in the cross-sectional dimensions suggests during BC production using the rotating-bioreactor, the bacteria extruded slightly more microfibrils per micropore, or the extruded microfibrils aggregated into larger structures (Newman 2008). The crystallite size of the BC produced in the static-culture was reported as 6.7 nm and 6.4 nm in the agitated-bioreactor by Czaja et al. (2004). These sizes are greater than those reported in the present study with static-culture at 4.9 nm and rotating-bioreactor at 5.6 nm. These differences could be a result of the difference in the strain used and/or the culture conditions.

The cellular movement and rotation around its longitudinal axis forms the ribbon assembly from the microfibrils. Therefore, the movement of cell is important for the determination of crystallization of either cellulose I or cellulose II as per the hypothesis of Hirai et al. (1997). The X-ray pattern with the highest peak between 20-25° suggested the presence of Cellulose I in both the BC samples produced in a rotating-bioreactor and the static-culture (Klemm et al. 2005). Since both the BC samples analysed showed the presence of cellulose I, it can be concluded that the cellular movement was similar in BC produced in both the static-culture and the rotating-bioreactor. The rotation of the cylinders did not impact the cell movement and the subsequent cellulose crystallization process. The cellulose I was composed of I α and I β but in order to determine the relative mass fractions CP/MAS ¹³C NMR analysis has to be conducted (Watanabe et al. 1998b). Although, NMR analysis was not conducted in the current study, it has been reported that the mass fraction of cellulose I α is related to the size of the BC crystallite microfibril (Yamamoto and Horii 1993; Yamamoto et al. 1996). It has been observed that the crystallites of BC produced in a rotating-bioreactor are smaller than that produced in the static-bioreactor, so it can be inferred that cellulose I β may be preferentially formed in the rotating-bioreactor although it needs to be confirmed

The crystallinity index was within the expected range of (60-90%) (Klemm et al. 2005). The crystallinity index of 89.4% estimated in the current study is comparable to a previous study of 89% between BC produced in the static-culture (Czaja et al. 2004). They also reported a drop in crystallinity index from 89% to 84% in the agitated-bioreactor that was similar to the drop in the rotating-bioreactor to 83%. The reduction in the crystallinity was attributed to the interference in the crystallization process as a result of agitation (Czaja et al. 2004). This hypothesis can be applied to the present study where there is a drop in crystallinity due to the agitation of the medium in the rotating-

bioreactor. In another study, the crystallinity index of BC produced in the static-culture was 81.65% compared to 67.17% in BC samples from an agitated-bioreactor (Yan et al. 2008). Yet another study reported the crystallinity index of BC produced in an agitated-bioreactor as 72% compared to that produced in static-culture as 80% (Watanabe et al. 1998b). These results too were consistent with a drop in crystallinity due to the agitation of the medium. There have been other reports of crystallinity of BC using a static-bioreactor as 85% (Cheng et al. 2009)

The X-ray diffraction was performed for the BC samples produced at different initial pH and glucose concentration of BC samples produced in a static-culture. Similarly, samples produced in a rotating-bioreactor at different tangential velocity, pH and initial glucose concentration were analyzed. There was no significant difference in either the dimensions of the crystals or the crystallinity index due to these parameter changes.

3.12. Summary

There is a structural difference between the BC produced in static-culture and in a rotating-bioreactor. This difference is not only observed at the macroscopic level but also observed at the crystallite level. The crystallinity index of the BC produced in the rotating-bioreactor was lesser than the BC produced in static-culture. These structural changes are probably due to the difference in size and distribution between the BC produced in a rotating-bioreactor and a static-culture as observed using a CSLM. There was little morphological change in the BC produced at different initial pH or glucose concentration produced in the static-cultures. A change in the cellulose ribbon width was noticed in the BC produced at different pH in the rotating-bioreactor alongwith the presence of macro-layers when produced without pH control. A difference in the level of scarring and orientation of layers was observed in the BC produced at varying pH using the rotating-

bioreactor. The reason for the change in morphology due to change in pH was not established.

CHAPTER 4

The influence of glucose concentration, pH and tangential velocity on BC production

4.1 Introduction

The BC production by any method largely depends on the strain of the bacteria, type and concentration of the substrate (saccharides) and the environmental parameters including oxygen availability, optimum pH and temperature. The growth rate and the subsequent production of cellulose are dependent on the bacterial strain (Schramm and Hestrin 1953) and the yield can vary from 0.003 to 0.3 g_{BC}/g_{glu} (Ishihara et al. 2002a). Before studying BC production using the RBC method, it was very important to determine a baseline for the strain used in the present study with respect to bioprocessing parameters. This was established using the static-culture method. This was the preferred method because of its simplicity and the availability of reports in literature for its comparative analysis. The agitated-bioreactor method too could have been used for the same, but the morphological nature of end-product did not support the material characterization methods used in this study. This is supported by reports that a more uniform pellicle that is acceptable in biomedical applications can be produced in a static-culture or using the rotating-bioreactor with cylinders (Setyawati et al. 2007). In the current study, the aim is to produce an end-product that can be used for biomedical applications.

The impact of variations of growth and culture conditions on the production efficiency of the strain in the static-culture method was estimated. These results were used for comparison with the rotating-bioreactor for analytical studies on BC production and its product characterization. The impact of different initial glucose concentrations and pH on the rate of production and yield were investigated in the static-culture including the influence of the walls of the culture vessel. While using the rotating-bioreactor, the

impact of varying initial glucose concentration, pH levels and tangential velocity on the rate of production and yield at a constant submersion level was investigated.

4.2 The different stages of BC production in the static-culture method

Understanding the stages for BC production provides insight into the cell growth that is linked to the BC production. The BC production in the static-culture method can be divided into the following four phases (1-4) adapted from Serafica (1997) and Watanabe et al. (1995) (Fig. 4.1).

Stage 1 – The inoculum containing viable cells of *G. xylinus* are transferred into the growth medium. There is a short lag phase (usually 48 hours) after which the cells enter the log phase. During this stage, the cells multiply and begin the cellulose production. The increase in the number of cells or cell growth is directly proportional to the cellulose production and it increases exponentially (Fig. 4.1).

Stage 2 – The continuous production of cellulose forms a layer at the air/liquid interface also known as BC film or pellicle (usually seen after 2-3 days after inoculation). Most of the cellulose producing cells gets entrapped in this film. The cell population in the film continues to rise along with the cellulose production. After the maximum cell density is reached, the cell number stops increasing. The cellulose production continues, leading to thickening of the BC pellicle perpendicular to the air/liquid surface area. Thus, cellulose production in the pellicle is almost linear with time due to a constant cell number at maximum cell density (Fig. 4.1).

Stage 3 – The cell and cellulose production continues at constant and maximum rate respectively, until one of the factors affecting the cell growth become limiting. Examples include substrate and/or oxygen mass transfer limitation due to thickening of the pellicle. This decreases the number of viable cells and the cellulose production rate (Fig. 4.1).

Stage 4 – The limitation of the substrate and/or oxygen and the rising pH due to the production of acetic and gluconic acids leads to, a decline in cell population, reducing the cellulose production until all the viable cells in the pellicle die and cellulose is no longer produced (Fig. 4.1).

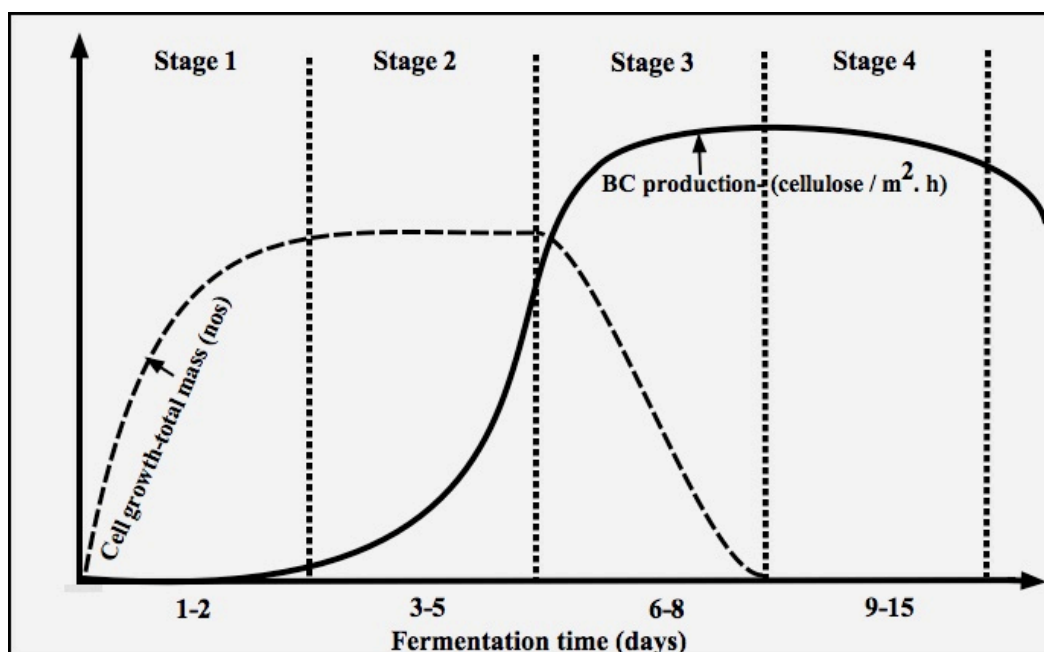


Fig. 4.1 – The different stages of cells growth and BC production observed in the static-culture are plotted on the x-axis. The interdependent rate of cell growth and BC production are plotted on the y-axis in the graph. Adapted from Serafica (1997).

The four stages were observed in static-cultures in this work. The cell growth was not directly estimated during the production of BC. It was indirectly estimated by the amount of glucose consumed due to the cellular activity in stage 1 and was usually observed to last between 1-2 days. The BC pellicle appeared between 3 to 5 days after inoculation and marked the beginning of BC production (stage 2). In stage 3, there was an increase in the thickness of the pellicle with the lower end moving into the medium while new layers

were added at the air-medium interface. The number of cells was calculated at harvest using the confocal microscope although for a few runs only. This stage lasted between 6-8 days. At stage 4, there was a decline in the rate of pellicle production (thickness) until it stopped, normally 9-15 days after inoculation.

4.3 Calculating the overall yield and rate of production of BC

The yield and the rate of production (R_p) were determined for each BC production method. The yield was defined as mass of BC produced (g) per mass of glucose consumed (g) reported as g_{BC}/g_{Glu} . The rate of production (R_p) was defined as weight of BC (g) produced per unit area of the surface of production (m^2) per unit time (day) (Appendix 4). It should be noted that since the lag phase was variable, it was corrected by taking $t = 0$ when the pH of the medium started dropping denoting the end of lag phase in the rotating-bioreactor. Since there was no set up to monitor the pH drop using the static-culture method, the $t = 0$ was recorded at the appearance of the BC membrane in the container.

Many investigations report yield as the mass of BC produced per unit volume (g/L) (Kim et al. 2007; Krystynowicz et al. 2002; Yang et al. 1998). This type of “yield” calculation is actually more closely aligned with the production rate defined in this work, because the value is the mass of cellulose produced over a certain period of time per unit volume of medium. The “yield” will change if the system is harvested earlier or later even assuming there are no changes in the underlying reaction system. Without accounting for time in the calculation, the “yield” is not an intensive value but extensive and therefore less useful as a description of system performance. Additionally, in most BC production methods, surface area, not volume, is considered the controlling variable (Masaoka et al. 1993; Serafica 1997) therefore surface area is a more suitable value to use than volume to

normalise for systems operated at different scales. In fact, reports in the literature generally show that for a static-culture, vessel volume does not affect “yield” (Masaoka et al. 1993; Okiyama et al. 1992a; Serafica 1997). If the vessel volume, surface area and harvest time are reported, traditional “yield” values can be converted to production rate as defined here. Throughout the present study, yield from published studies have been recalculated as defined for comparative analysis when data permits.

4.4 The influence of the wall of the culture container used the static-culture method known as “wall-effect”

The rate of production in the static-culture method is affected by the cell density, sugar (substrate) and its initial concentration and the surface-area of the medium at optimum growth conditions (Masaoka et al. 1993). Quite recently, another parameter affecting the rate of production in the static-culture was introduced known as the “wall effect” by Hornung et al. (2006). This effect was explained as the frictional forces exerted by the walls of the container on the growing pellicle that opposed the sinking of the BC pellicle into the growth medium (BC density > glucose medium). Using different shaped culture flasks, they demonstrated an increase in rate of production in a conical shaped flask, wherein the wall effect was reduced to zero when the pellicle moves downwards and was no longer in contact with the walls. This report refuted the past reports by Iguchi et al. (2000). They reported that during the static-culture assays performed in their group, initially the BC pellicle failed to form in conical shaped flasks, because there was no adhesion of the BC to the wall, which was necessary for formation of a continuous layer. They were in agreement on BC moving downwards into the medium during the course of growth.

While this theory was not tested in different shaped flasks (glass), a similar result was noticed in vertical walled McCartney glass bottles. Approximately 5 % of the bottles had a non-uniform diameter and resembled the flasks with the ID increasing from the liquid surface to the bottom of the bottle. These bottles with non-uniform inner diameter (20-22 mm) produced pellicles that filled-up nearly 45% of the bottle and used up the entire medium for the growth (Fig. 4.2). The bottles where the inner diameter was uniform had pellicle growth that filled about 10-20% of the bottle and remained afloat on the medium (Fig. 4.2). Our observation was similar to the BC produced in the conical flasks made by Hornung et al. (2006). These observations support the theory that the rate of production of BC can be influenced by the wall effect. This theory was further tested as discussed in the following sections.



Fig. 4.2 - BC production in McCartney bottles using the static-culture method. The left bottle has a regular inner diameter of 20 mm and has BC pellicle in the medium. The right bottle has an irregular inner diameter that increased from 20 to 22 mm and the pellicle has used up the entire medium for its growth and filled approximately 45% of the bottle.

4.5 BC production in the static-culture method

Bacterial cellulose production in the static-culture is directed by the total number of cellulose producing cells in the growth medium (Marx-Figini and Pion 1974). Hence, a constant amount of inoculums was maintained throughout the static-culture cultures in proportion to their volume. BC was produced in the static-culture using the same volume of medium (100 ml) but different surface area (at air/medium interface) of 15 and 32 cm² (sample size 10 for each surface area). Similar fermentation conditions were maintained for 5 days (excluding lag phase which was similar for most of the cultures) for each of the samples. The initial pH of the medium was constant for all the cultures at 4.54 but the pH of the medium at harvest varied between 2.98 to 3.10.

The rate of production increased directly proportional to the surface area of the air/liquid interface from an average of 7.00 ± 0.98 to an average of 12.00 ± 1.58 g_{BC}/m²·day (mean \pm standard deviation, sample size =10). The rate of production nearly doubled when the surface area was doubled. But this increase in production rate did not have a significant impact on the yield that increased only marginally from an average of 0.094 ± 0.020 to an average of 0.11 ± 0.08 g_{BC}/g_{Glu} (mean \pm standard deviation, sample size =10). This result was in accordance with earlier published reports (Masaoka et al. 1993; Okiyama et al. 1992b). They also reported that the volume of the medium did not affect the amount of BC produced.

4.5.1 Difference in the rates of production of BC observed in the static-culture method

In order to investigate whether the increase in the rate of production was due to the influence of surface area, “wall effect” or due to any other factor including growth stage, further assays were conducted. The assays with surface area 32 cm² (sample size 10) were

repeated and the fermentation time was doubled to 10 days in order to change the stage of growth at harvest. The cell density of inoculums, amount of substrate and oxygen and growth conditions (pH and temperature) were also similar. There was a difference in the rate of production which can be visually inferred from the difference in thickness of the BC pellicles visible through five of the 10 transparent bottles shown (Fig. 4.3). There was a difference in the rate of production within the cultures fermented for 10 days and ranged from 8.6 to 17 $\text{BC}/\text{m}^2\cdot\text{day}$ while the average was $13 \pm 3 \text{ BC}/\text{m}^2\cdot\text{day}$ (mean \pm standard deviation, sample size =10). The yield ranged between 0.09 to 0.15 $\text{g}_{\text{BC}}/\text{g}_{\text{Glu}}$ with an average yield of $0.11 \pm 0.023 \text{ g}_{\text{BC}}/\text{g}_{\text{Glu}}$ (mean \pm standard deviation, sample size =10). The fermentation time was same for all the cultures (10 days). The variable lag phase could have contributed to the difference in the rate of production and yield. Since the lag phase was based on the appearance of BC membrane (subject to human error) unlike in a rotating-bioreactor where the lag phase was based on the drop in pH monitored online. This could also be the result of the “wall effect” wherein difference in friction between the pellicle and wall of the container may have influenced the rate of production (Hornung et al. 2006). This hypothesis would require further investigation.

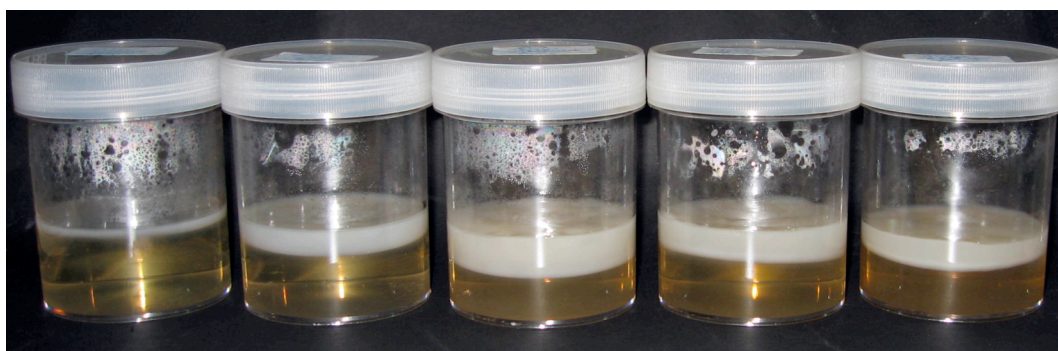


Fig. 4.3 - A sub-set of five out of the ten polypropylene bottles (ID = 45 mm volume = 200 ml glucose concentration = 50 g/L) with, BC pellicle of varying thickness. For every

culture, 100 ml of growth medium was used and similar set of culture and growth conditions were maintained for these static-cultures.

4.5.2 Discussion

The average rate of production of BC using the static-culture method was estimated at $13 \text{ g}_{\text{BC}}/\text{m}^2\cdot\text{day}$ at glucose concentration of 50 g/L which is comparable to $10.62 \text{ g}/\text{m}^2\cdot\text{day}$ as per the reports by Serafica (1997) at similar concentration of glucose. It is also similar to the rate of production between $12 - 17 \text{ g}/\text{m}^2\cdot\text{day}$ as reported by Borzani (1995). The average yield estimated in the present study was $0.12 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ and is comparable to the reports in literature. A yield between the ranges of $0.03 - 0.06 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ in static culture was reported by Bodin et al. (2007a) while Toda et al. (1997) reported the yield as $0.15 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ in an acetic acid-resistant strain of *G. xylinus*. The relatively small dissimilarities between the rate of production reported in the current study to that reported in literature could be either due to “wall effect” and/or the difference in strain or both. The difference in the yield in various studies could be due to the different strains used and / or due to the difference in the amount of gluconic and acetic acid produced as a by-product and will be discussed in greater detail in section (4.10). Another point to consider with respect to the yield is that the difference in the rate of production, formed pellicles of different thickness in the culture bottles (Fig. 4.3). This difference in thickness could have a direct impact on the diffusion path of the substrates from the medium. This can cause a difference in the yield of the BC produced despite using the same strain and similar conditions of culture and growth. Additionally it has recently been published that the BC production in a static-culture is dependent on the fermentation period. This group reported the maximum yield of $0.2 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ after 144 hours of fermentation for the strain using corn steep liquor as the medium (El-Saied et al. 2008).

4.6 Bacterial cellulose production using a rotating-bioreactor

After having established a baseline for the yield and productivity, the same was investigated for BC production using a rotating-bioreactor. The parameters that influence the uptake of substrate and oxygen are crucial to BC production. The advantage of the rotating-bioreactor method over the static-culture method is that in the rotating-bioreactor method the amount and flow of substrates can be controlled while maintaining the desired pH and temperature.

4.6.1 The different stages of BC production in the rotating-bioreactor method

The BC production in a rotating-bioreactor is similar to that of the static-culture. Both the methods depend on the initial density of cells used as inoculate and the surface area available for the BC production. The difference lies in the fact that in a rotating-bioreactor, theoretically, there would be little or no limitation of carbon substrate and oxygen uptake due to diffusion. This is because the surface of the cell-bearing pellicle is exposed to the liquid medium and air alternately. This ensures that the pellicle in the rotating-bioreactor grows indefinitely as long as the substrate and oxygen is continually supplied, assuming all other growth parameters including pH and temperature are favourable. This is compared to the static-culture, where the liquid substrate and oxygen diffuse from opposite sides of the pellicle, thereby eventually limiting production. In addition, it is very difficult to control the pH in a static-culture and the continuing drop in pH can also eventually become inhibiting. Thus the BC production in a rotating-bioreactor method can be summed up in the following two stages as adapted from Serafica (1997).

Stage 1- The inoculum is added to the growth medium in the rotating-bioreactor while maintaining favourable conditions for dissolved oxygen, pH and temperature. After a

short lag phase, the cell density in the medium increases and eventually produces a cellulose film coating on the rotating cylinders. The number of cells in this film increases until maximum cell density is reached increasing the thickness of the film or pellicle (Fig. 4.4).

Stage 2- The cellulose pellicle grows linearly while the total number of cells per unit volume remains constant at all times. Cellulose film growth can be maintained indefinitely as long as adequate nutrients and oxygen are supplied to the surface of the film. This maximum cell density is maintained as long as regular supply of oxygen and nutrient is available at favourable pH and temperature in aseptic conditions (Fig. 4.4).

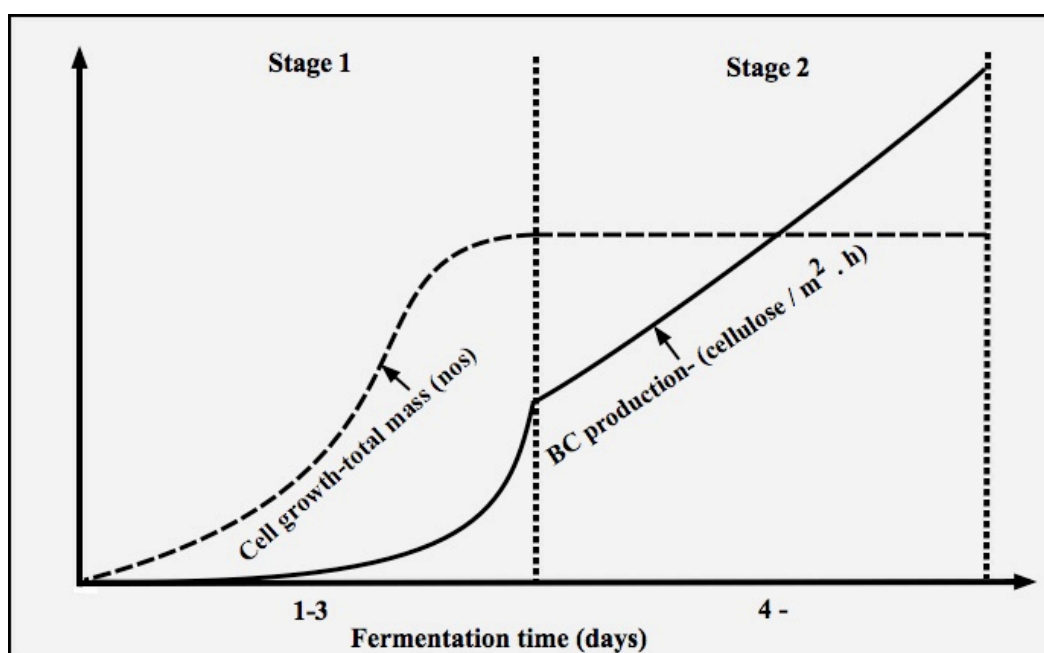


Fig. 4.4 - The different stages of cell growth and BC production observed in the rotating-bioreactor. The interdependent rate of cell growth and BC production are on the y-axis in the graph. Adapted from Serafica (1997).

4.6.2 Production of BC using a rotating-bioreactor

The growth medium in the rotating-bioreactor was inoculated and after a short lag phase (about 12 hrs), the cell concentration increased and eventually some cells attached to the felt on the cylinder surface. While the increase in the number of cells was not measured directly, it was inferred from the drop in the initial pH of the medium. This pH drop was because of the co-production of acids such as gluconic and acetic along with BC. They continued to increase exponentially and in due course formed a BC film on the rotating cylinders. In most of the fermentation runs, the BC membrane formation on the cylinders was noticed within 48-72 hrs after the inoculation. The exception was in runs without active pH control where the pellicle appeared after 80-96 hours. The cells in the BC film coating the cylinder continued to grow in the film and produced BC simultaneously thereby increasing the thickness of the pellicle. This consequently increased the thickness of the BC film perpendicular to the growth surface in accordance with Borzani and De Souza (1995). The BC pellicle that formed on both the cylinders was used for all the analysis including assessment of yield, rate of production and the various material characteristics (Fig. 4.5). The rate of production is not influenced by the production stages because there is no definite end to the second stage unless the oxygen and /or substrate is limiting

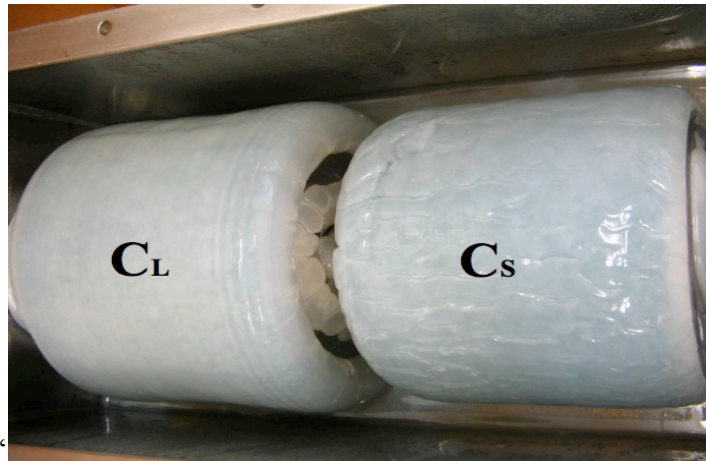


Fig. 4.5 - The BC pellicles formed on the two cylinders of the rotating-bioreactor: the large cylinder (C_L) with a diameter = 140 mm and the small cylinder (C_S) with diameter = 120 mm.

4.6.3 Summary of the fermentation runs completed in the rotating-bioreactor

In total, 63 runs were conducted during the entire course of study (Table 3). About 38% of the fermentation runs were lost due to various reasons including mechanical failure but the most common cause was contamination and it occurred most of the times in runs controlled above pH 6.0.

Table 3- Summary of the fermentation runs completed.

Description of the runs	Total (no)
Runs controlled at pH 4.0	16
Runs controlled at different pH from 2.5 – 6.0	7
Runs without pH control	6
Runs controlled at different glucose concentrations	8
Runs with co-polymers	2
Successful runs	39
Failed runs	24

For all the runs, the initial glucose concentration was set at approximately 50 g/L (except those investigating the different concentrations of sugar), to ensure that the substrate was not limiting during the course of the run. The longest run was conducted for 16 days and it did not run out of sugar. The typical initial pH for most runs was 4.5 and the run time was approximately 6 days.

Although total surface area available for bacterial growth and subsequent BC production on both the cylinders combined was 0.098 m², BC was produced on the non-cylinders areas of the reactor as well in the reservoir. This BC on non-cylinder areas was referred to as slop. This additional BC was accounted for in the total yield. It is difficult to calculate the amount of sugar utilized to produce the pellicles on the two different cylinders individually. Hence, the total yield of both the cylinders was a summation of both the pellicles recorded as the weighted average tangential velocity of the cylinders, along with the BC produced on the non-cylinder surfaces or slop. The pH was maintained at 4.0 ±

0.4 (mean \pm standard deviation, sample size =16) for all the runs (except those investigating the influence of pH) with the addition of caustic, as BC was reportedly produced favourably at this pH. Additionally, the highest oxygen uptake occurs at pH 4.0 (Embuscado et al. 1994;Masaoka et al. 1993;Verschuren et al. 2000).

Previous reports suggest that the volume and depth of the medium do not influence the productivity of BC (Masaoka et al. 1993). Carbon substrate uptake is an important factor to be considered in the growth and production of any bacteria and for *G. xylinus* oxygen uptake is of equal importance because of its aerobic nature. In the rotating-bioreactor method, diffusion of the oxygen and substrate takes place through the exposure of the growing pellicle to the air and medium alternately. Thus, the amount of time the pellicle is submerged in the medium is of prime importance. This can be impacted by the level of submersion of the cylinder. At submersions less than 50%, the cylinder spends more time in the medium than in the air and if greater than 50% submersion, the opposite is true. Thus, a study on the impact of variation of submersion level on the production of BC was investigated. Another factor that can influence the length of time the pellicles remain submerged in the medium is the rotations per minute (RPM) which is converted to tangential velocity in this study. While the exposure of the substrates to the growing pellicle is important, the concentration of the substrates also plays a vital role in the diffusion of the same. Hence, the influence of the varying concentration of glucose is an important factor for investigation. The *G. xylinus* bacteria requires a pH between 4.0-5.0 for good growth and production of BC (Embuscado et al. 1994;Hestrin et al. 1947), hence the study of pH was crucial from point of cellulose production. While the oxygen uptake is also crucial to this obligate aerobe, it was not investigated in this study and a steady supply of oxygen was provided to all the fermentation runs carried out in this study.

4.6.4 Diffusion of substrates during submersion and aeration

The different parts of the growing biofilm are exposed to medium and oxygen alternately during the rotation; therefore, it is important to understand the how the diffusion of substrates takes place. The mass transfer during the aeration phase is different than that in the submerged phase. When the cylinders are submerged in the medium, the concentration of substrates in the medium is higher than that within the biofilm because of microbial metabolism. This difference in concentration drives the substrates (glucose and oxygen) from the medium into the biofilm. The concentration gradient is a function of time, thus the substrates continue to diffuse in the biofilm until equilibrium is attained which will depend on the length of time the biofilm is submerged. The bacterial cells in the biofilm consume these substrates and if the rate of diffusion is the same as the rate of consumption, their concentration in the biofilm remains constant.

Using the biofilm characteristics, Serafica (1997) calculated the oxygen penetration depth of approximately 875 μm before its consumption by the bacterial cells. The oxygen penetration depth when the pellicle was submerged was found to be lesser (263 μm) as the oxygen concentration in the bulk liquid is about 30% of the saturated value. Thus most of the sugar is obtained by the pellicle when it is submerged while it gets maximum oxygen during aeration (Serafica 1997).

4.7 Influence of difference in submersion level

The influence of submersion level on the BC production and yield was initially investigated for the same strain by Schrecker (2004). This work was a transitional phase; hence a series of runs was conducted at different submersion levels before proceeding with the study of the other parameters. The results presented are a combination of the new data obtained and that of Schrecker.

The cylinders C_L and C_S rotated at 7 and 6.16 RPM respectively due to the difference in their diameters, which represented a tangential velocity of 0.05 m/s and 0.044 m/s for the surface of each cylinder respectively. No cellulose production was observed at 100% submersion. There was a trend of fall in rate of production with an increase in the submersion of the cylinders the highest rate of production ($4.62 \text{ g/m}^2\cdot\text{day}$) was recorded at 26 % submersion and the lowest ($1.29 \text{ g/m}^2\cdot\text{day}$) at 87 % (Fig. 4.6). The rate of production was scattered but constant below 50 % submersion and declined sharply with further increase in submersion. The average yield was $0.12 \text{ g}_{BC}/\text{g}_{glu}$ at lower levels of submersion (10 - 28 %) and $0.08 \text{ g}_{BC}/\text{g}_{glu}$ at higher levels of submersion (44 - 83 %) (Fig. 4.7).

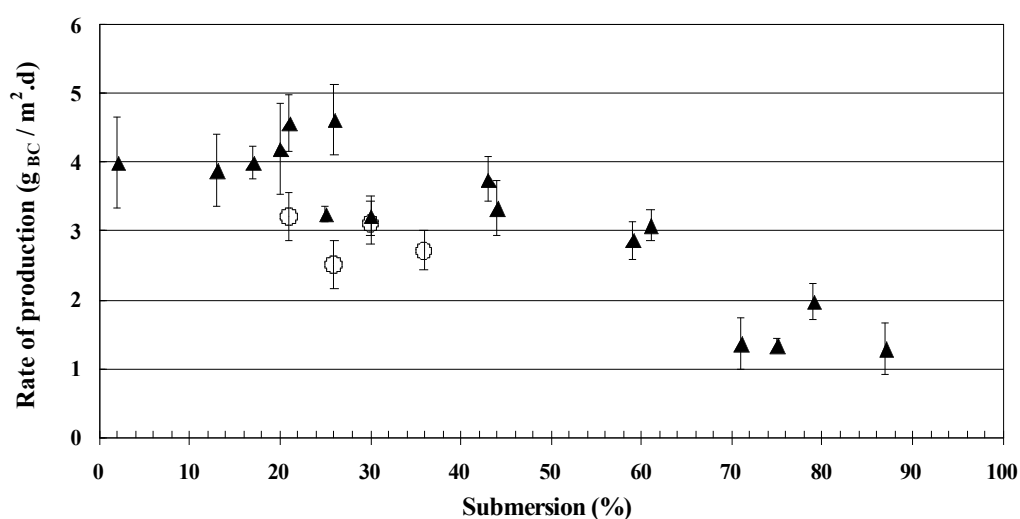


Fig. 4.6 - Rate of production of BC produced in a rotating-bioreactor at different levels of submersion. The error bars represent variability between the dry weight of the BC samples (sample size=10) obtained from each of the two cylinders (Schrecker 2004). The data from the current study is also included (open circles).

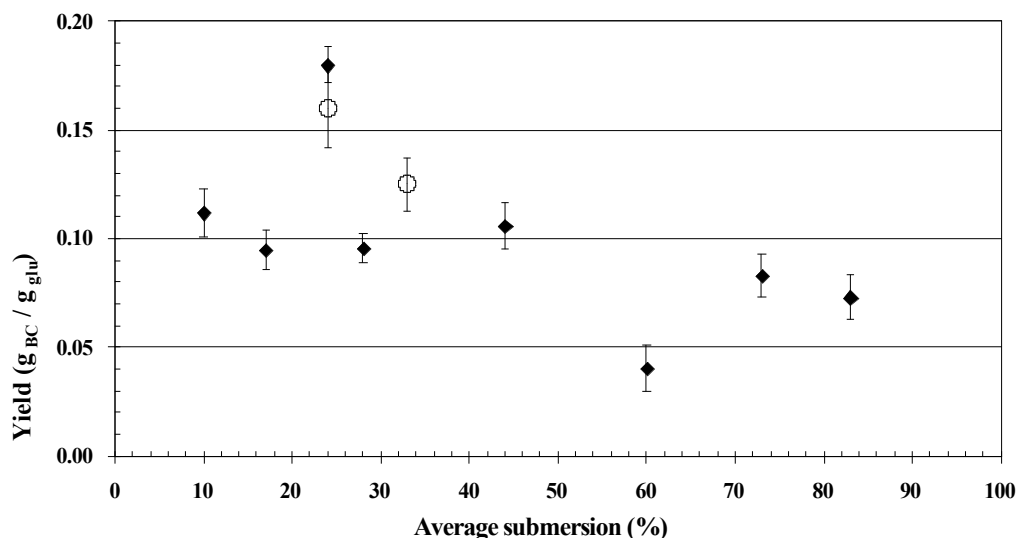


Fig. 4.7 - The yield of BC produced at an average submersion level of the two cylinders. The variability represents the difference between the weights of the dry BC (sample size=10) from each cylinder (Schrecker 2004). The data from the current study is included (open circles). (The data from the Shrecker was recalculated because of a calculation error).

4.7.1 Discussion

The decreases in both the rate of production and yield with increase in submersion level was non-linear, with a strong trend of overall reduction in rate of production and yield with an increase in submersion. The rate of production decreased to nearly one fourth from 4.62 to 1.29 g/m²·day when the submersion was increased from 26% to 87% (Fig. 4.6). The average yield was 0.12 g BC/g glu at lower levels of submersion (10 - 28 %) and 0.08 g BC/g glu at higher levels of submersion (44 - 83 %) (Fig.4.7). All the fermentation runs had more than 0.5 g/L glucose at harvest which rule out the possibility of glucose limitation (Serafica 1997). The lower yield and rate of production at increased submersion could be a direct consequence of the reduced aeration. It has been reported that the BC production from glucose is a function of the supply of oxygen (Hestrin et al.

1947). They reported negligible BC production under a nitrogen atmosphere and about half the amount of BC in air and the maximum BC production were observed in 100% oxygen. This was supported further by another report that suggested that inadequate supply of oxygen can lead to production of smaller quantities of cellulose (Kato et al. 2007). The lowest yield at 0.04 g BC/g glu was observed at 60% submersion level (Fig. 4.7). Although the result of the rate of production and yield are scattered and non-linear, there is a tendency for lower BC yield at higher levels of submersion. There should be more data points (at least 3 runs per submersion level) to arrive at definitive conclusions.

4.8 The influence of glucose concentration on the production efficiency of BC in the static-culture method

The growth of *G. xylinus* and production of BC is influenced by the type of substrate and its concentration (Embuscado et al. 1994). The effect of initial glucose concentration was investigated using the static-culture method for initial glucose concentrations from 2-100 g/L. For each glucose concentration, five samples were produced with an equal total fermentation period of 12 days (lag + exponential). BC production was not observed in any of the five cultures produced at an initial glucose concentration of 100 g/L. The appearance of BC film marked the end of lag phase and the beginning of exponential phase varied at different initial glucose concentrations. The exponential phase for most concentrations was 10 days except 57 g/L (9 days), at 72 g/L (8 days) and at 87 g/L (7 days) respectively.

There was an increase in the yield from 0.16 to 0.24 g BC/g glu with the increase in initial glucose concentration from 2 to 11 g/L after which there was a drop in the yield to 0.14 gBC/gglu with a further increase in glucose concentration of 21.75 g/L (Fig. 4.8). Again, there was a steady increase in yield from 0.17 to 0.196 g BC/g glu at a glucose

concentration of 39 and 72 g/L respectively after which there was a drop in yield to 0.120 at 87 g/L (Fig. 4.8).

The rate of production showed a trend similar to that of the observed in the yield. There was an increase in rate of production from 0.63 to 14.17 g_{BC}/m²·day with an increase in glucose concentration from 2 to 48 g/L (Fig. 4.9). There was a drop in the rate of production from 48 g/L and the lowest rate of production 2.03 g_{BC}/g_{glu} was recorded at 87 g/L (Fig. 4.9).

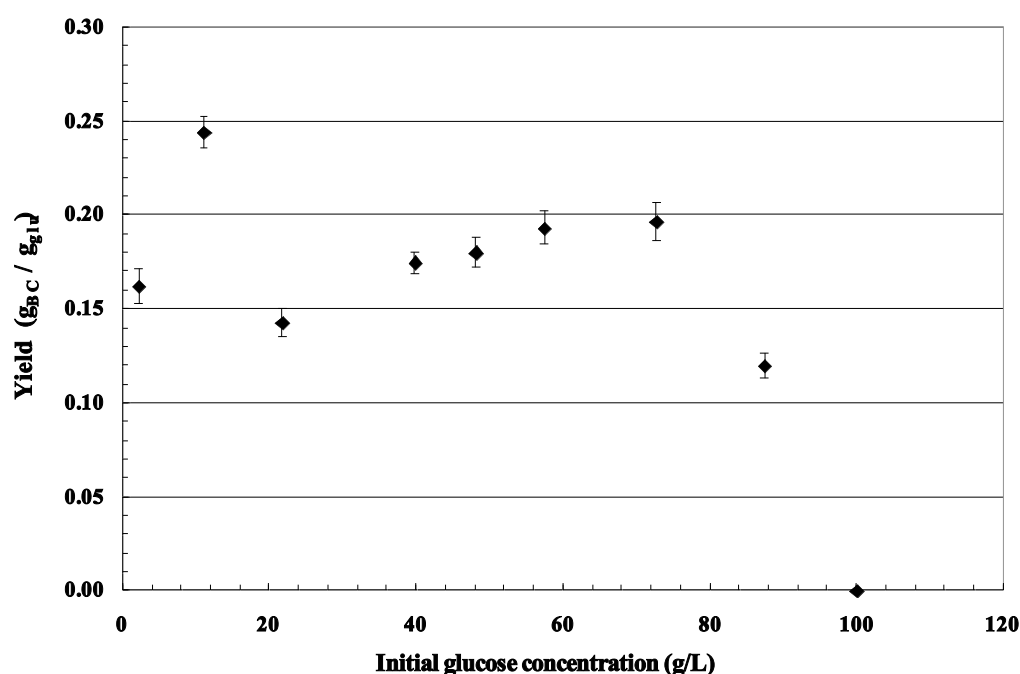


Fig. 4.8 - The yield of BC produced in the static-culture method for 10 days with different initial glucose concentrations. The variability represents the difference between the glucose concentrations of the BC (sample size = 40 with 5 replicates for each concentration). The cultures were produced in polypropylene bottles (ID = 45 mm and volume = 200 ml) for each of the different glucose concentration.

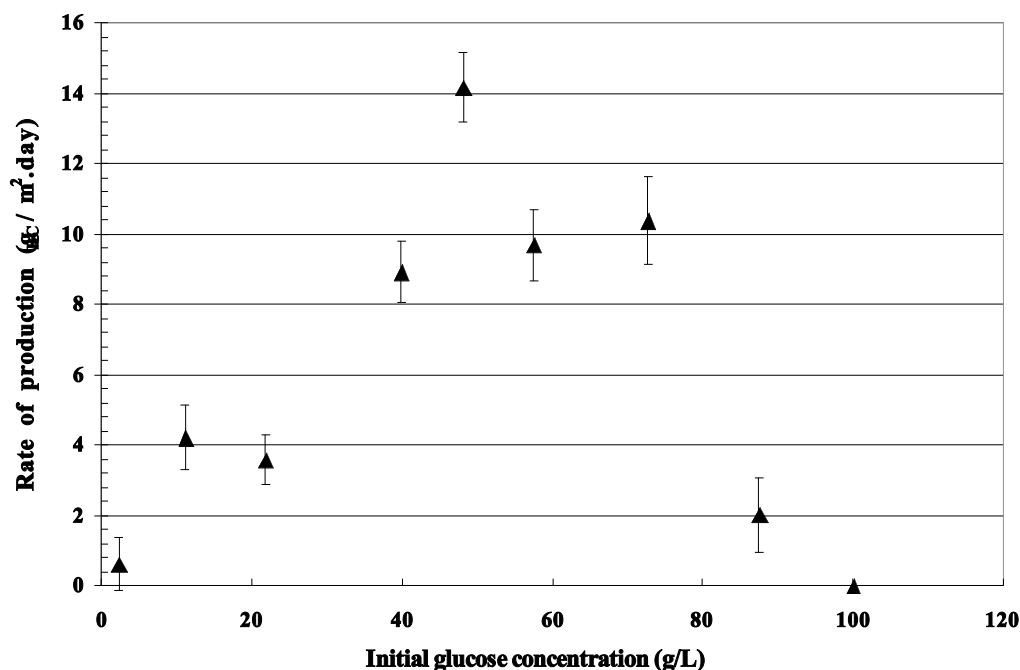


Fig. 4.9 – The rate of production of BC produced in the static-culture for 10 days (excluding lag phase) at different initial glucose concentrations. The variability represents the difference in the dry weight between BC samples (sample size = 40) from each of the 5 replicates produced in polypropylene bottles (ID = 45 mm volume = 200 ml at the eight different glucose concentrations).

4.9 Acid production

The synthesis of cellulose in *G. xylinus* can occur via two alternative pathways (Ogawa and Tokura 1992). The direct phosphorylation of exogenous glucose is one of the pathways. The indirect gluconeogenesis pathway via the pentose cycle is the other one (Ross et al. 1991). Some glucose is converted into gluconic acid by the glucose oxidase in the cell membrane and the resulting acid is released into the medium. Other oxidation products such as keto-gluconic and acetic acid are also produced in addition to gluconic acid (Hestrin and Schramm 1954a). It is therefore necessary to analyse the amount of acid

produced at during BC production at different initial glucose concentrations. This will give a better picture of the impact glucose concentration on yield.

4.10 Influence of acid production on the bioprocessing efficiency of BC produced in the static-culture at different initial glucose concentration

The acid produced in all the cultures was analyzed using HPLC (materials and methods, section 2.16.2). This analysis was conducted in order to determine the impact of acid production, if any, on the rate of production and yield of BC at different initial glucose concentration. Acetic acid was produced in all the cultures at different glucose concentrations, but gluconic acid was produced at higher glucose concentrations of 21 and 87 g/L (Fig. 4.10). Maximum gluconic acid 182 mM was produced at an initial glucose concentration of 57 g/L (Fig.4.10). The least amount of gluconic acid 0.9 mM was produced at 11 g/L while no gluconic acid was produced at the initial glucose concentration of 2 g/L (Fig. 4.10). While maximum amount of acetic acid 80 mM was produced at 21 g/L and the least 4 mM at an initial glucose concentration of 72 /L (Fig. 4.10).

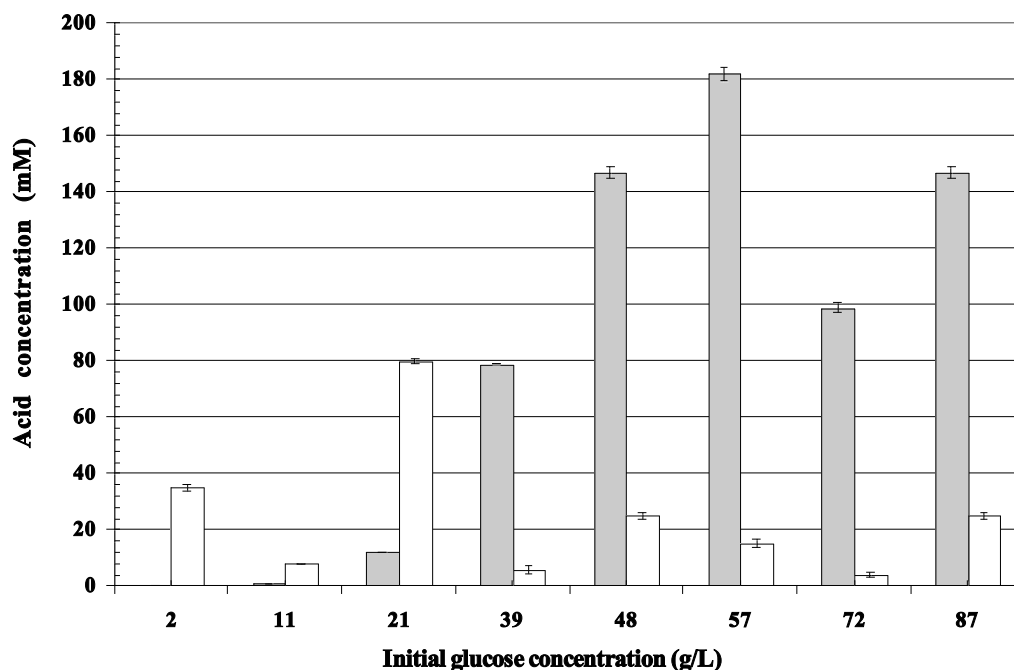


Fig. 4.10 - The average amount of gluconic (grey bars) and acetic (white bars) acid produced (mM) in the medium in the static-culture at different initial glucose concentrations. The variability represents the difference in the glucose concentration between each of the medium samples (sample size = 5) for each of the different glucose concentrations.

The carbon mass balance was estimated for better analysis of results. In the current study, cell biomass and the rate of constant CO₂ emission was not evaluated and were grouped as one. The results were normalised by calculating the number of moles of carbon utilized from glucose to produce BC and both gluconic and acetic acid. The carbon not accounted for in these products was assumed to be utilized for production of biomass and carbon dioxide. It was found that at lower concentrations of glucose 2-21 g/L there was greater production of cell mass and cellulose and lesser production of acid (Fig. 4.11). In contrast, the excessive substrate at higher concentrations of glucose (39-87 g/L) was directed towards acid production (Fig. 4.11).

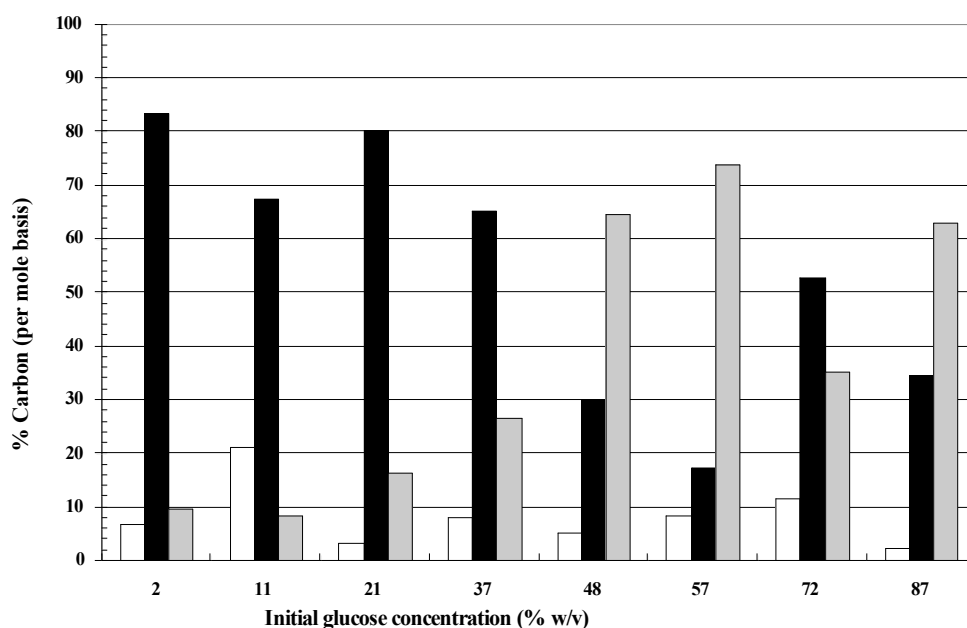


Fig. 4.11 - The amount of carbon (moles) utilized for the metabolic products such as, cellulose (white), gluconic and acetic acid (grey) and carbon dioxide and biomass (black) at different initial glucose concentration in the static culture.

4.10.1 Discussion

The increase in the initial glucose concentration should lead to an increase in the rate of production because greater amount of glucose availability should translate into greater amount of cellulose. Although there was an increase in the rate of production with an increase in the glucose concentration from 2 to 48 g/L, there was a drop in the same when the concentration increased further from 57 to 87 g/L (Fig. 4.9). The yield did not exactly co-relate with the rate of production but it followed a similar trend at higher glucose concentration. There was an increase in the yield from 0.16 to 0.24 g_{BC}/g_{glu} with the increase in initial glucose concentration from 2 to 57 g/L (Fig. 4.7). This could be because of an improvement in cellulose production due to higher rate of sugar concentration (Ishihara et al. 2002b) or enhanced substrate diffusion. After a point if the same pathways are active at all times the yield eventually reaches a constant value when

the rate of diffusion of substrate and rate of cellulose synthesis reach equilibrium. Additionally alternative pathways could be used to produce acid. A study reports that higher amount of gluconic acid was produced preferentially at higher concentrations of glucose (Yang et al. 1998). The acid concentration estimates of the current study where the minimum acid production was at 11 g/L and highest acid production at 87 g/L supports the report. The yield estimates complements the acid production as the highest yield was observed at 11 g/L and the lowest at 87 g/L (Fig.4.8).

Masaoka et al. (1993) conducted similar assays at glucose concentrations between 5 – 40 g/L in static-culture. They reported a constant rate of production of 36 g_{BC}/m²·day but a reduction in yield from 0.1 g_{BC}/g_{glu} at glucose concentration of 5 g/L to yield of 0.028 g_{BC}/g_{glu} at 40 g/L concentration of glucose. They suggested that the decrease in yield was due to the production of gluconic acid. The lower rate of production in the current study could probably be attributed to “wall effect” and the reported steady rate of production reported by Masaoka et al. could be absence of the same. On evaluating the amount of acid produced by the different glucose concentrations, it was found that at higher initial glucose concentrations more gluconic acid was produced. There was a fluctuation in the rate of production of acetic acid but no co-relation with the initial glucose concentration was observed. The increase in gluconic acid production with glucose concentration has been reported by various researchers. Masaoka et al. (1993) reported that the excessive production of gluconic acid at higher initial glucose concentrations did not impact cellulose production. The reports of Schramm et al. (1957a) agrees with the observations of Masaoka et al. but suggests that although gluconic acid does not impact cellulose production directly, the reduction in the pH level due to the same hinders it to some extent. In another related study, it was found that there was an increase in the rate of BC production when *G. xylinus* strains producing lesser gluconic acid were used (White and

Brown 1989). The reason for the same was not very clear and there was ambiguity between a probable impact on cell division and/or influence on the biosynthetic pathway of cellulose (White and Brown 1989). The result from the current study supports the hypothesis of Schramm et al. (1957b) as the pH was not controlled in these assays and there was an observed reduction in yield with an increase in gluconic acid. This hypothesis was tested in similar assays under controlled pH using a rotating-bioreactor and the results are discussed in the proceeding sections.

It was also observed that there was a difference in the rate of utilization of glucose at different glucose concentrations. More glucose was consumed per hour (0.048 - 0.078 g/h) at lower glucose concentrations (2 - 39 g/L) as compared to (0.014 - 0.038 g/h) at higher concentrations of glucose (57 - 87 g/L) (Appendix 5). This was opposed to the logic where one expects more glucose to be consumed at higher concentrations than vice versa. Considering that similar growth conditions were maintained for all the cultures the difference in the rate of production and yield of BC was attributed to the variation in the concentration of substrate. The probable reason for the same was the preferential production of gluconic acids at higher initial glucose concentrations.

4.11 The influence of initial glucose concentration on the production efficiency of BC produced using a rotating-bioreactor

The influence of initial glucose concentration was investigated in the rotating-bioreactor. Different initial glucose concentrations (8-87 g/L) were investigated at the same submersion level (26% for large cylinder and 21% for the smaller cylinder), average tangential velocity (0.095 m/s (7 RPM for the large cylinder and 6.14 for the small cylinder due to the difference in the cylinder diameter) and pH (4.0). The rate of production was highest (4.8 g_{BC}/m²·day) at the initial glucose concentration of 10.5 g/L

(Fig. 4.12). But at a higher initial glucose concentration, of 77 g/L there was a steep drop in rate of production to $1.24 \text{ g}_{\text{BC}}/\text{m}^2\cdot\text{day}$ (Fig. 4.12). Trends comparative to the static-culture were observed in the rotating-bioreactor in both the rate of production and yield at varying initial glucose concentrations. The yield initially increased from 0.1 to $0.3 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ with the increase in the initial glucose concentration from 8.8 to 26 g/L (Fig. 4.13). There was a drop in yield to $0.0134 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ with further increase in the initial glucose concentration to 77 g/L (Fig. 4.13).

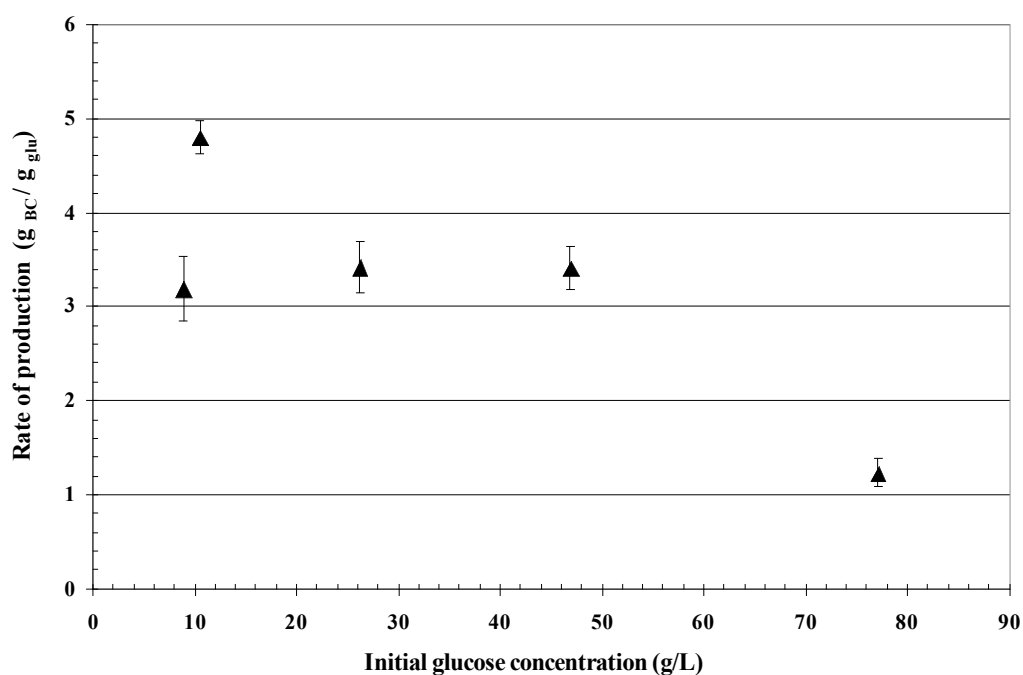


Fig. 4.12 - The average rate of BC production produced at different initial glucose concentrations using the rotating-bioreactor. The variability represents the difference in the dry weight between the BC samples (sample size = 10) from the pellicle of each cylinder produced at different glucose concentrations.

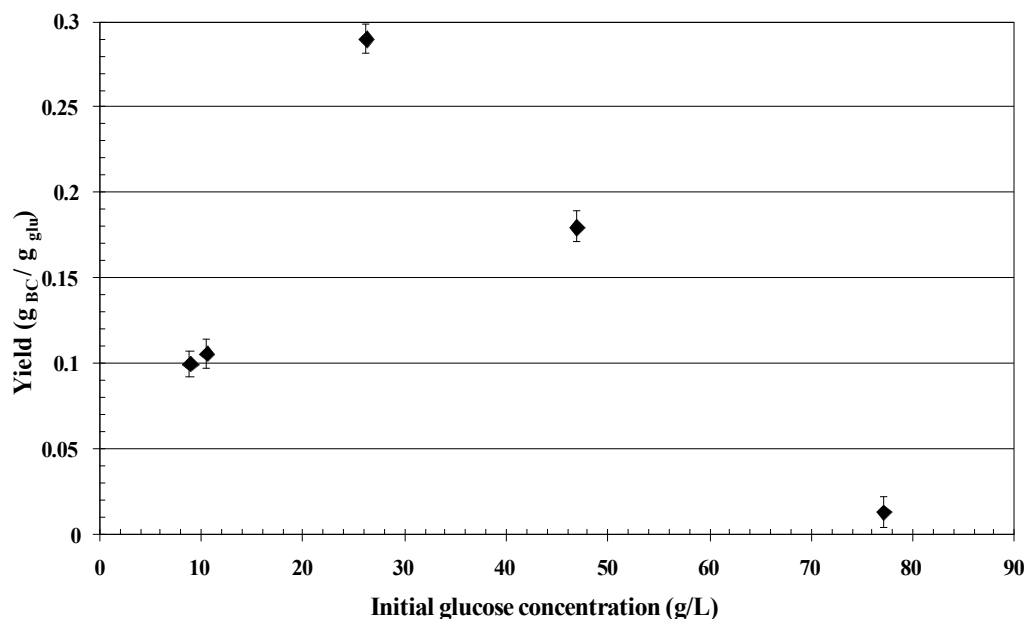


Fig. 4.13 - The average yield of BC produced at different initial glucose concentrations in the rotating-bioreactor. The variability represents the difference in the glucose concentration between the medium samples (sample size=10) for different glucose concentrations.

In static cultures, it was observed that at higher initial glucose concentration a greater amount of acid is produced that consequently lowered the yield. Thus the medium in the rotating-bioreactor was analyzed for the presence of acids using HPLC. Similar to the static-culture, acetic acid was produced in all the cultures at different initial glucose concentrations (8 -77 g/L). The concentration of acetic acid increased from 19 to 255 mM with an increase in glucose concentration from 8-77 g/L (Fig. 4.12). Higher amounts of acetic acid (maximum = 250 mM) was produced in the rotating-bioreactor as compared to the static-culture (maximum=80 mM) (Fig. 4.15). The gluconic acid concentration too increased linearly from 56 to 209 mM from 8 to 46 g/L but then reduced to 77 mM with a further increase in glucose concentration to 77 g/L (Fig. 4.14). The gluconic acid too was produced in greater amount in the rotating-bioreactor (maximum = 209 mM) than in the static-culture (Fig. 4.10).

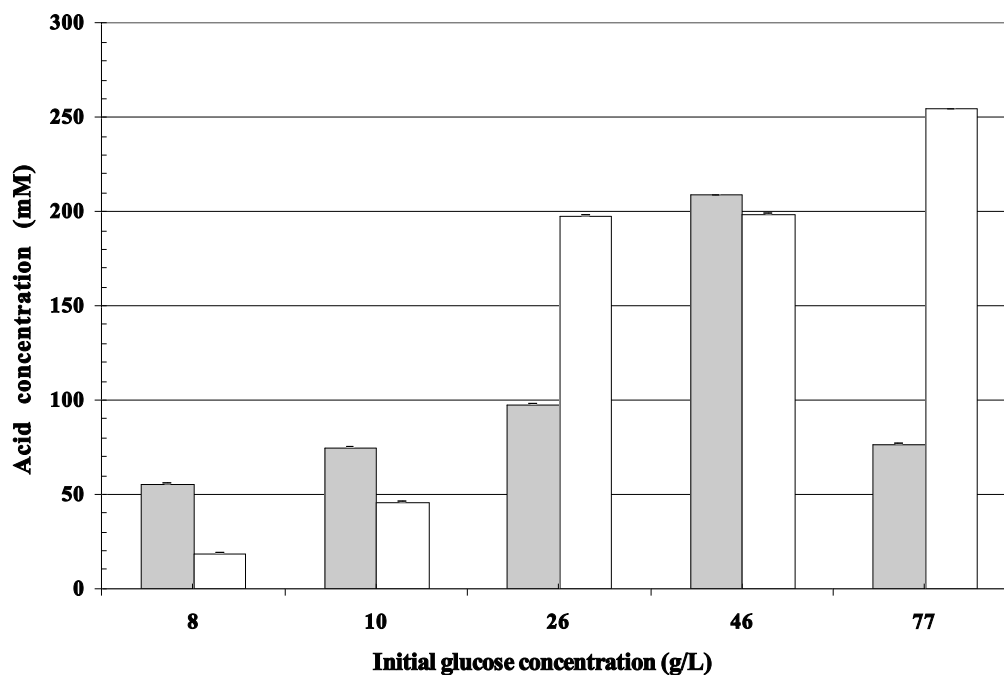


Fig. 4.14 – The average amount of acid produced at different concentrations of glucose using the rotating-bioreactor (grey-gluconic acid and white-acetic acid). The variation represents the difference in the amount of acid from the medium samples (sample size=3) produced at different glucose concentrations.

4.11.1 Discussion

There are not many reports in the literature of BC production at varying glucose concentrations using a rotating-bioreactor. The different initial glucose concentration investigated by Serafica (1997) using a rotating-bioreactor were 0.05 to 1.05 g/L which is very low compared to the concentrations tried in the current study. He reported the highest rate of production at 0.05 g/L, but it would be difficult to compare the results because the rate was calculated per unit thickness of the pellicle. He also suggested the use of not more than 0.05 g/L of glucose for higher yield with lower concentrations of gluconic acid. The actual yields obtained at different glucose concentrations and the total acid produced was not reported.

In the present study, the highest rate of production was observed at a glucose concentration of 10.5 g/L whereas, Krystynowicz et al. (2002) reported the same at 20 g/L. This may be because the difference in the rate calculation $\text{g}_{\text{BC}}/\text{L}$. The highest yield of $0.24 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ was obtained at the concentration of 10.5 g/L while Krystynowicz et al. (2002) reported the highest yield of $0.4 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ at a concentration of 5 g/L. Results and observations similar to this study were reported by Hwang et al. (1999) albeit in agitated cultures. They reported an overall reduction in cell growth, rate of production and yield with the increase in glucose concentration. The maximum yield of $0.21 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ was reported at 10 g/L and the lowest $0.13 \text{ g}_{\text{BC}}/\text{g}_{\text{glu}}$ at 40 g/L. It is worth noting that at higher concentrations of glucose the glucose consumption rate is higher (1.2-1.6 g/h) at higher initial glucose concentrations of 46 to 77 g/L as compared 8-26 g/L (0.28 - 0.4 g/h) (Appendix 5).

Although, acetic acid was produced in the static-culture at varying glucose concentrations it did not follow any trend. However, the acetic acid production in the rotating-bioreactor was directly proportional to the increase in the initial glucose concentrations. In addition the larger amounts of acetic acid (maximum = 255 mM) was produced in the rotating-bioreactor compared to the static-culture (maximum=80 mM). The results from current study suggest that greater amount of acid is produced in the rotating-bioreactor as compared to the static-culture during BC production. This could be directly related to the increased exposure of oxygen to the growing pellicle in the rotating-bioreactor leading to an increase in oxidative products like acids. The results from the current study also does not support the hypothesis of published reports (Masaoka et al. 1993;Schramm et al. 1957a). As per their reports, the cellulose production is hindered by the falling pH due to increased production of gluconic acid and not because of the presence on the acid alone. In the current study, the pH was controlled at a favourable level of 4. Despite the pH

control, a low yield of 0.0134 g_{BC}/g_{glu} was observed at an initial glucose level of 77 g/L. It should also be noted at this glucose concentration the gluconic acid was only 0.077 mM whereas the highest gluconic acid of 209 mM was produced at 46.9 g/L and the yield was much higher at 0.18 g_{BC}/g_{glu}. The highest yield was observed at 26 g/L while the gluconic acid production was 98 mM. The current study hints towards some other parameter affecting the cellulose production at higher concentrations of sugar. These assays should be repeated with more data points in at least triplicates for each of the glucose concentration to confirm the results and further investigation should be carried out to find the causal factor for the same. In order to rule out the influence of the method of production different glucose concentration assays should be repeated by controlling the pH in a static-culture.

The rate of glucose consumption in a rotating-bioreactor was approximately ten times higher than that observed in the static-culture (Appendix 5). This difference could be due to the increase in the production of gluconic acid as a consequence of increased aeration caused due to the rotating cylinders. The glucose utilization trend in the rotating-bioreactor was opposite to that observed in the static-culture at different initial glucose concentrations. At lower glucose concentrations (8.87- 26.2 g/L), glucose was consumed at a proportionately lesser rate of 0.46 - 0.28 g/h. While at higher glucose concentrations of 46 and 77 g/L, the glucose consumption rate increased to 1.22 and 1.63 g/h respectively (Appendix 5). The growth conditions were similar in both the static-culture and the rotating-bioreactor in addition to the volume of inoculums and the area for cellulose production. The reason for this reversal in glucose consumption rate at different glucose concentration cannot be explained without the measurement of dissolved O₂ during BC production and should be a subject for further study

4.12 The influence of different tangential velocity on the production efficiency of BC produced in the rotating-bioreactor

Along with the submersion level, the speed of rotation of the cylinders also influenced the duration of submersion of the cylinder in the medium. The influence of tangential velocity or RPM on the BC production was investigated. The rotational speed was reported as tangential velocity to account for the cylinders having different diameters. Varying velocities was one of the reasons for using cylinders instead of discs for this research, as the tangential velocity was far more constant during BC production facilitating analysis. With discs the tangential velocity varied with diameter.

The study of BC production at varying submersion levels showed that the maximum rate of production was observed when the big cylinder was maintained at (C_L) 26% and the small cylinder (C_S) at 21% level of submersion in the medium (Schrecker 2004). Therefore, for all subsequent investigations this was the selected submersion level. Two cylinders of different radii (60 and 70 mm) were used. Hence, their tangential velocities differed, giving two results at each RPM for the rate of production. However, as yield was calculated using the bulk glucose concentration, only one yield was reported for each RPM tested and the tangential velocity was the average of the two cylinders.

The tangential velocity was varied between 0.013 to 0.16 m/s (2 RPM - 22 RPM). Fermentation runs were not carried out at tangential velocities greater than 0.16 m/s, as the pellicles developed cracks and were not suitable for further investigations. At tangential velocities lower than 0.013 m/s, no BC production was observed on the cylinders. The rate of production increased proportionally to the tangential velocity ranging from approximately 1.75 to 4.0 g_{BC}/m²·day over the range of tangential velocity (Fig. 4.15). The yield initially increased as the tangential velocity varied from 0.01 - 0.09

m/s, peaking at 0.18 g_{BC}/g_{glu} and decreasing to 0.09 g_{BC}/g_{glu} as the tangential velocity increased to 0.15 m/s (Fig. 4.16).

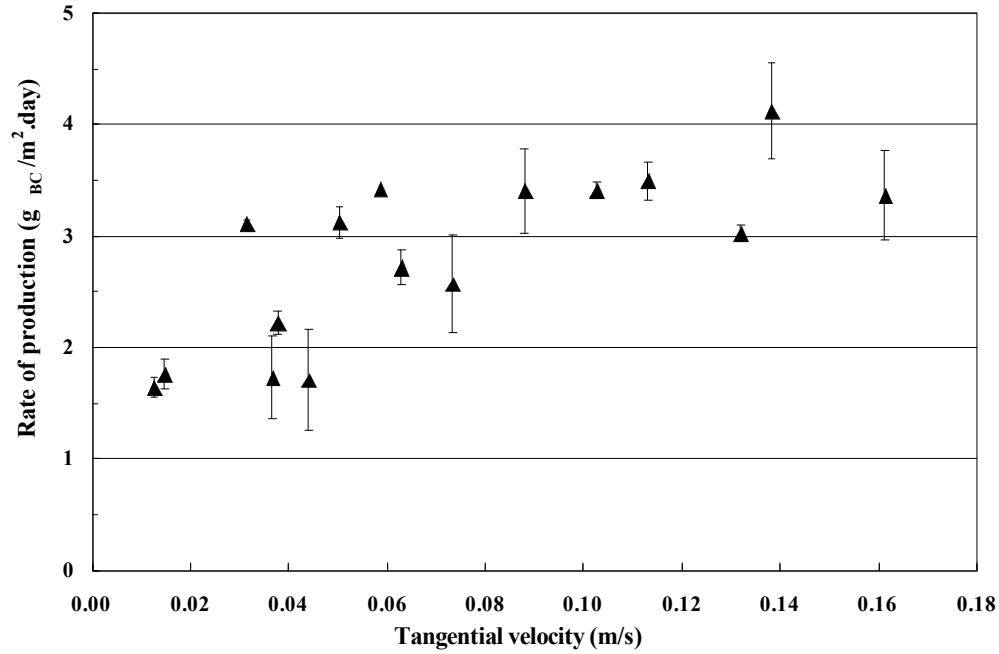


Fig. 4.15- The rate of production of BC using a rotating-bioreactor at different tangential velocities. The variability represents the difference between the dry weight of BC samples (sample size = 10) produced at different tangential velocities.

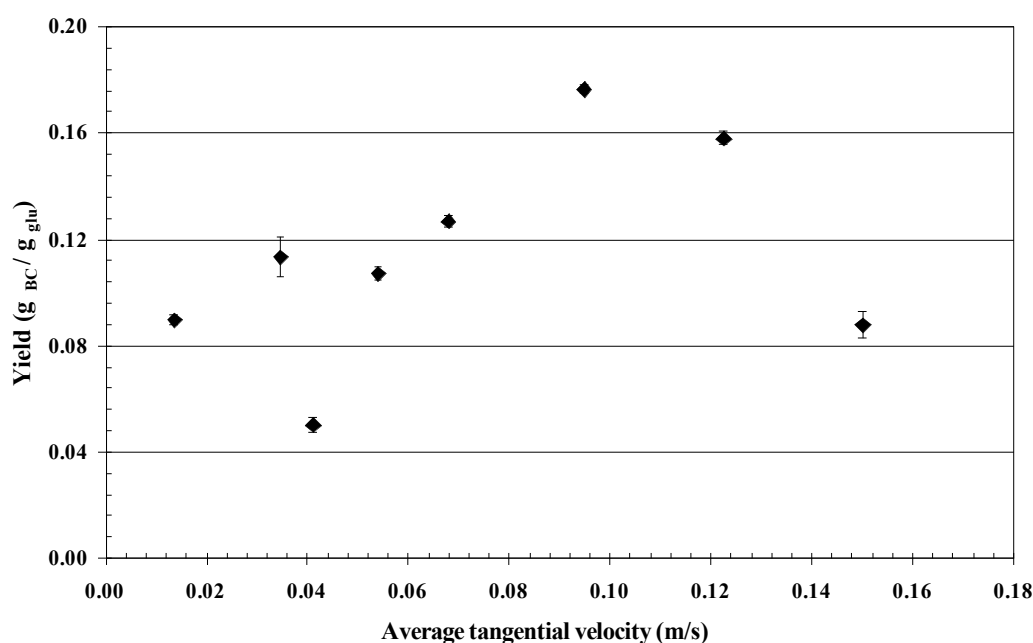


Fig. 4.16 - The yield of BC obtained at different average tangential velocities using two cylinders in a rotating-bioreactor. The variability represents the difference between the glucose concentration of medium samples (sample size = 3) at different tangential velocities.

4.12.1 Discussion

BC was produced at different tangential velocities but it was observed that no BC was formed at tangential velocities lower than 0.013 m/s. This could be due to the requirement of a certain amount of liquid film on the cylinder surface for the initial formation of the cellulose film. At very low tangential velocity, as the cylinder exits the medium, the medium tends to run off gradually, leaving no liquid layer for initial film formation (Serafica 1997). The increase in tangential velocity increased the rate of production almost linearly (Fig. 4.18). There is a trend of increase in the rate of production almost proportional to the increase in tangential velocity. Watanabe and Yamanaka (1995) altered the rate of BC production by controlling the oxygen tension at different levels in

the static-culture. Similarly, the current study reports the control of the rate of BC production in a rotating-bioreactor by varying the tangential velocity of the cylinders.

The BC yield has been reported to be directly proportional to the rate of oxygen transfer (OTR) and the coefficient of oxygen transfer (K_{La}) (Kouda et al. 1997b). Thus, the increase in tangential velocities would have increased the K_{La} giving a higher yield. But after a point when the tangential velocity increased there was a reduction in yield (4.16). This reduction in yield could be due to several reasons. The increase in tangential velocity also leads to the increase in thickness of the pellicle (Serafica 1997). This could lead to the reduction in the coefficient of mass transfer of both the substrate and the oxygen because these rates reduce with increase in film thickness due to increased mean path length (Famularo et al. 1978). The study conducted by Serafica (1997) using rotating-bioreactor confirmed that the increase in the speed of rotation or tangential velocity decreased the mass transfer coefficient that consequently led to a reduced oxygen transport across a given area. Serafica (1997) also suggested the reduction in the tangential velocity after film formation, as a probable solution for the same. Another reason could be an increase in cellulose non-producing mutants due to increased agitation (Kouda et al. 1997b; Verschuren et al. 2000). It has been reported by Kim et al. (2007) that the cell concentration increased with an increase in tangential velocity but the cell type (BC-producing or non-producing) was not differentiated. Alternatively, it could be due to the loss of substrate due to direct oxidation (Kouda et al. 1997a). Thus the reduction in yield at higher tangential velocities could be due to any one of the reasons mentioned or a permutation and combination of any of them. To get an accurate reason for the difference in yield at different tangential velocities the different parameters such as oxygen transfer rate (OTR), coefficient of oxygen transfer (K_{La}), coefficient of mass

transfer for both the substrates and the number of viable cells both cellulose producing and non-cellulose producing should be recorded during the fermentation.

While an increase in the rate of production with the increase in tangential velocity was observed during this study, a decrease in the same was reported by Kim et al. (2007). They reported the rate of production as $7.6 \text{ g}_{\text{BC}}/\text{m}^2\cdot\text{day}$ at 0.09 m/s (15 RPM) that reduced to $6.3 \text{ g}_{\text{BC}}/\text{m}^2\cdot\text{day}$ with an increase in tangential velocity to 0.22 m/s (35 RPM). Their results are not in agreement with the study and this could be due to the difference in the experimental design and set up and /or the total fermentation time. They used discs at 34% submersion level whereas in the present study cylinders were used and the submersion level was 50%,. In case of discs, the tangential velocity is the average of different points on the radius whereas on a cylinder, it is fairly constant only varying slightly as the pellicle increases in thickness. Besides, assays were not conducted beyond 0.18 m/s and therefore there are lesser data points for comparison. Additionally, their estimates were based on fermentation runs completed in 4 days whereas; the average fermentation run in the study was 6 days of cellulose production.

4.13 Influence of acid production on the bioprocessing efficiency of BC production using the rotating-bioreactor at different tangential velocities

The lowering of the observed BC yield with an increase in tangential velocity could also be due to production of acids like gluconic and acetic and/or increase in the number of non-cellulose producing mutant cells. Therefore, it is necessary to evaluate the amount of acid produced before inferring about the influence of tangential velocity on the yield of BC. Acetic acid was produced at all the tangential velocities except at the highest tangential velocity of 0.16 m/s . There was a trend towards reduction of acetic acid from 88 to 0 mM with the increase in the tangential velocity from $0.013 - 0.16 \text{ m/s}$ (Fig. 4.17).

Although the highest amount of acetic acid 95 mM was observed at 0.43 m/s (Fig. 4.17). There was a trend of reduction in the gluconic acid production 40 - 15 mM with an increase in the tangential velocity (Fig. 4.17). The highest amount of gluconic acid 85 mM was observed at 0.10 m/s (Fig. 4.17).

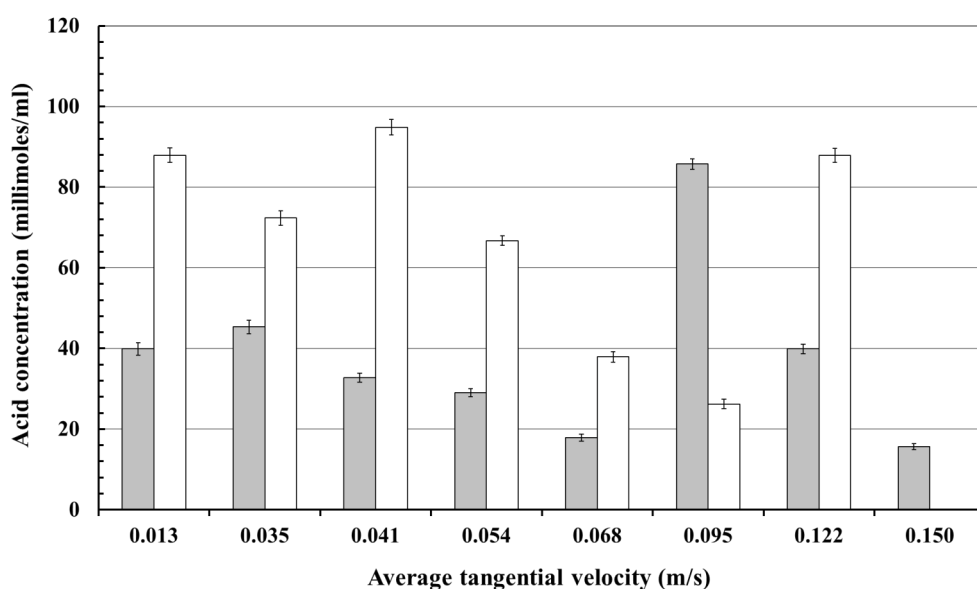


Fig. 4.17 -The average amount of gluconic acid (grey) and acetic acid (white) produced during BC production using a rotating-bioreactor at different tangential velocities. The variability represents the difference in the amount of acid between medium samples (sample size = 3) produced at different average tangential velocities.

The data from the acid production did not show any clear trends that would influence the yield and rate of production at different tangential velocities. Additionally, it was reported that the increase in the tangential velocity led to an increase in the number of non-cellulose producing mutant bacterial cells. The number of cells and the rate of CO₂ emission were not evaluated in these fermentation runs but for some indication, the carbon mass balance was evaluated. The results were normalised by calculating the

number of moles of carbon utilized from glucose to produce BC and acids (gluconic and acetic). The carbon not accounted for in these products was assumed to be utilized for production of biomass and carbon dioxide. The amount of metabolised carbon that ended up in acid production reduced from 78% at 0.013 m/s to 14% at 0.15 m/s with the exception of 43% at 0.122 m/s (Fig. 4.18). The amount of biomass and carbon dioxide increased from 15% at 0.013 m/s to 56% at 0.068 m/s and then reduced to 47% at 0.122 m/s (Fig.4.18). The highest amount of carbon converted to biomass and carbon dioxide was observed at the highest tangential velocity of 0.15 m/s (Fig. 4.18).

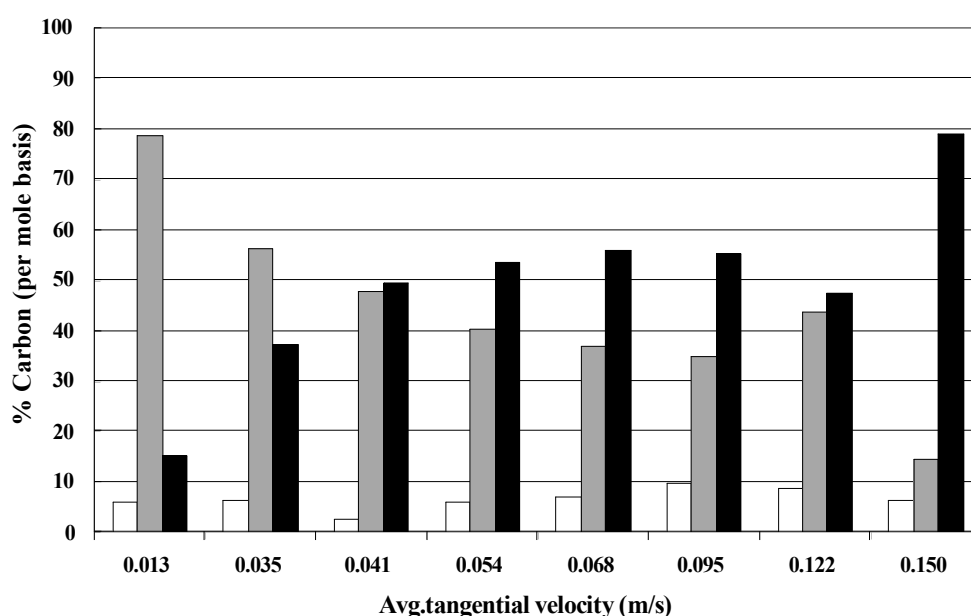


Fig. 4.18 - The amount of carbon (moles) utilized for the metabolic products such as, cellulose (white), gluconic and acetic acid (grey) and carbon dioxide and biomass (black) at different average tangential velocities.

4.13.1 Discussion

Although no clear relationship can be established between the yield and the amount of acid produced, some observations are worth highlighting. The maximum acid but minimum biomass was produced at the lowest tangential velocity and the maximum biomass but lowest amount of acid was produced at the highest tangential velocity under study (Fig. 4.18). The increase in biomass could be due to the increase in the non-cellulose producing mutants because despite the decrease in acid, the BC yield was low. Although the number of cellulose producing cells and non-cellulose producing mutants have not been identified and counted in the current study due to non-availability of set-up, this conclusion was based on published reports by Kim et al. (2007). Hwang et al. (1999) reported an increase in the number of cellulose non-producing mutants with an increase in the RPM. Additionally, there was an increase in yield by four times with the increase in RPM by about three times. This research was conducted in an agitated culture which much greater agitation. In order to get a good understanding of the impact of difference in tangential velocity direct measurement of CO₂ and biomass production is required. The cells should also be tested to determine whether they are cellulose producing or non-producing cells.

4.14 The influence of pH on the production efficiency of BC

The pH of the growth medium is one of the vital factors in bacterial growth and the synthesis of polysaccharides (Embuscado et al. 1994). The influence of pH on the BC production in a rotating-bioreactor was investigated by controlling the medium at different pH values including 3.0, 3.5, 4.0, 5.0 and 6.0. BC production was not observed at pH 2.75 and below, and due to contamination issues; fermentation runs above pH 6.0 were not performed. The success rate of producing BC at pH 6.0 was about 10% because of the increased frequency of contamination at this pH. The custom apparatus used in the

present study made it more susceptible to contamination than a commercial fermentor. All the fermentation runs were conducted at an average tangential velocity of 0.095 m/s and at submersion level of 26% for the larger cylinder C_L and 21% for smaller cylinder C_S . These experiments were compared to runs without active pH control (no pH control). A typical run without pH control had a pH range between 4.5 ± 0.1 to 2.6 ± 0.4 (mean \pm standard deviation, sample size = 3).

The yield reduced with an increase in pH except at pH 3.0. The highest yield was 0.66 g BC/g_{glu} in the no pH control, while the lowest yield of 0.06 g BC/g_{glu} was observed at pH 6.0 (Fig. 4.19). The yield at pH 4.0 and 5.0 was not very different at 0.114 and 0.12 g BC/g_{glu} respectively. The same trend did not appear in the rate of production. The highest rate of production was observed at pH 3.0 and pH 5.0 at 3.78 and 3.54 g $BC/m^2 \cdot day$ respectively and the lowest 2.28 g $BC/m^2 \cdot day$ at pH 6.0 (Fig. 4.20).

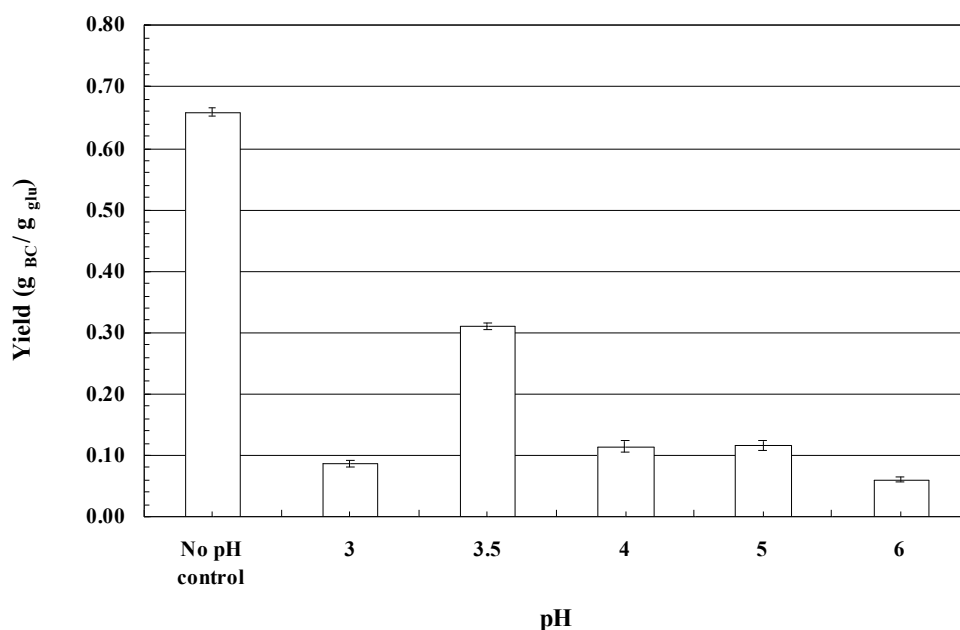


Fig. 4.19 - The yield of BC produced in a rotating-bioreactor controlled at different pH. The variability represents the difference between the glucose concentrations at harvest

between medium samples (sample size = 10) produced at different pH values (tangential velocity = 0.095 m/s, initial glucose concentration = 50 g/L).

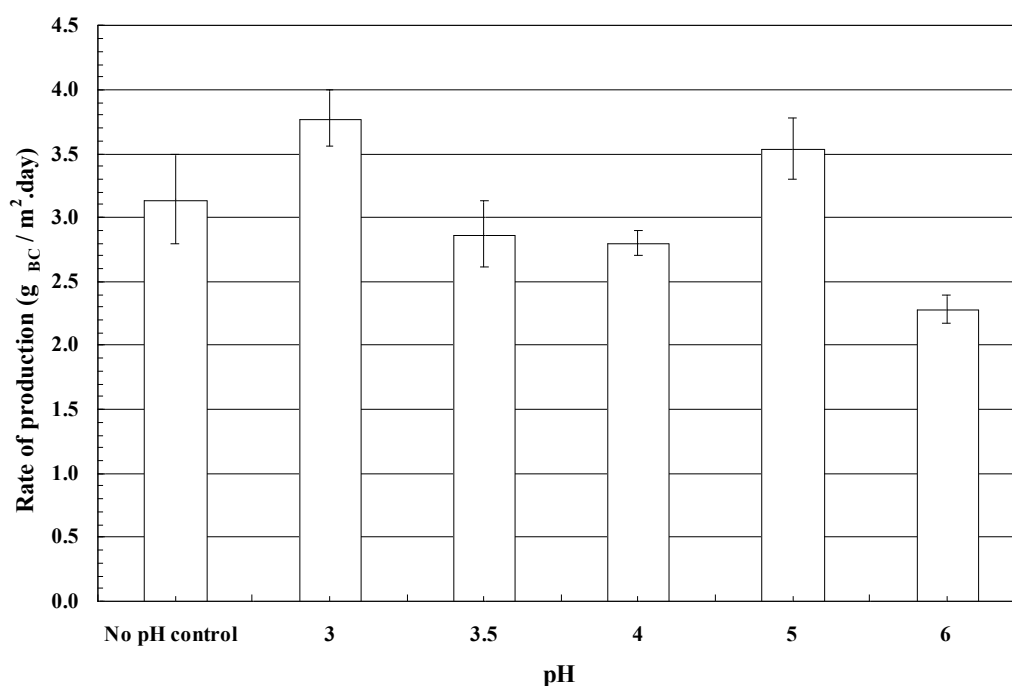


Fig. 4.20 - The rate of production of BC produced in a rotating-bioreactor controlled at different pH. The variability represents the difference in the dry weight of the samples (sample size = 10) produced at different pH values and without pH control (tangential velocity = 0.095 m/s, initial glucose concentration = 50 g/L).

4.14.1 Discussion

Kim et al. (2007) investigated the influence of a range of pH (5.0-8.0) on BC production in a rotating-bioreactor and found the highest rate of production in the runs where the pH was not controlled (the initial and final pH was not mentioned) and lowest at pH 6.0. The rate of production was reported in g/L, hence was recalculated with the available data as $7.8 \text{ BC} / \text{m}^2 \cdot \text{day}$ and the lowest $3.82 \text{ BC} / \text{m}^2 \cdot \text{day}$ at pH 6.0. There was insufficient data for calculation of yield hence the comparison with the result obtained was not possible. Besides there were fewer data points for comparison since, while they tested the influence

of pH 5.0, 6.0, 7.0 and 8.0 this work was conducted at the pH 3.0, 4.0, 5.0 and 6.0. Additionally, the fermentation time differed from being only 4 days in their work compared to an average of 6 days for this work. Hwang et al. (1999) observed the influence of pH (4.0, 5.0 and 6.0) in agitated cultures and found that pH 5.0 was most suitable for both cellulose growth and production but pH 4.0 was favourable for conversion of glucose to gluconic acid and subsequently BC. They reported a yield of 0.20 BC/g_{glu} at pH 5.0 compared to 0.15 BC/g_{glu} at pH 4.0 and pH 6.0. They confirmed that glucose was favourably converted into gluconic acid at pH 4.0 that explained the lowered BC production. The study of Hwang et al. (1999) supports the lower rate of BC production at pH 4.0 in the present study as compared to pH 3.0 and no pH control. Again, the comparison of result is difficult because they did not report the yield of fermentation runs at pH 3.0 or in no pH control. Additionally, they used the agitated-bioreactor for BC production while in the current study a rotating-bioreactor was used. In the present study, acetic acid (0.1 N) was added to the medium that was approximately at pH 4.5 in order to reduce the pH to 3.0 and 3.5. This could have led to an increase in the rate of BC production and yield at pH 3.0 and 3.5 in the present study as observed in the study of kombucha (Lee et al. 2002). The reason for enhanced BC production was not mentioned. The rate of BC production and the yield was greater when the pH was not controlled in the rotating-bioreactor as compared to the runs with pH control. This observation is in accordance with Chao et al.(2000).

4.15 The influence of acid production on the bioprocessing efficiency of the BC produced at different pH using a rotating-bioreactor

Acid analysis at different pH values and without pH control showed that acetic and gluconic acid varied (Fig. 4.21). There was an increase in the amount of gluconic acid production from 14.15 to 85.73 mM from pH 3.0 to 4.0, which was also the highest

production (Fig. 4.21). It then reduced to 22.55 and 10 mM at pH 5.0 and 6.0 respectively. Similar trends were noticed in acetic acid production except that the highest of 104 mM was recorded at pH 3.5 (Fig. 4.21). It should be noted that the acetic acid added for maintaining the pH has been accounted for in the various runs.

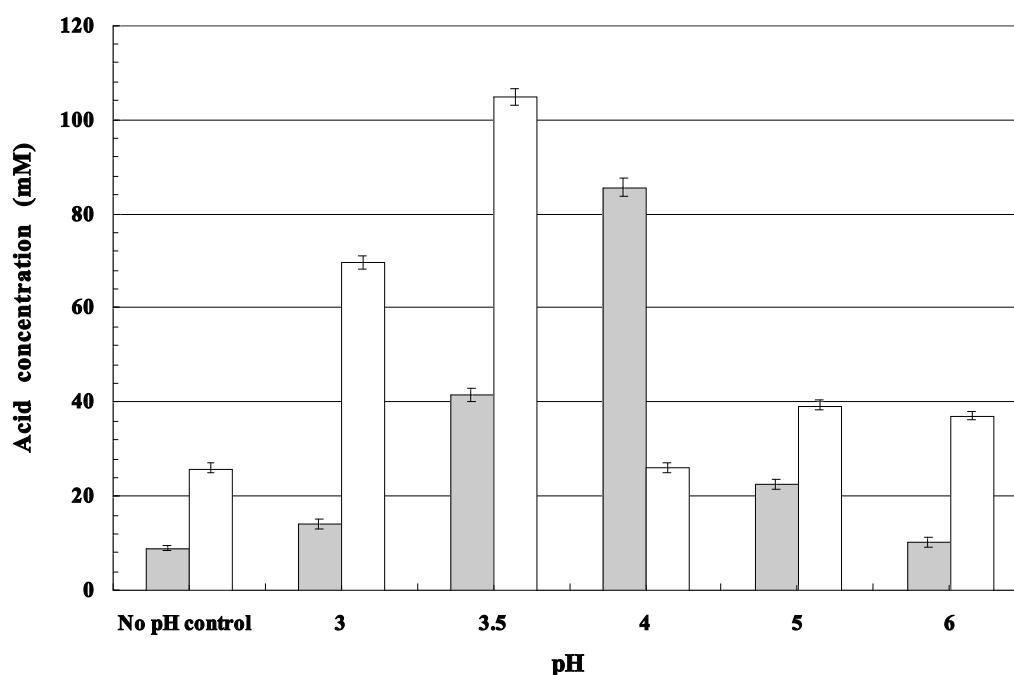


Fig. 4.21 - The average amount of gluconic acid (grey) and acetic acid (white) produced BC production using a rotating-bioreactor at different pH and without active pH control (no pH control). The variability represents the difference in the acid concentrations between medium samples (sample size=3) produced at different pH.

4.15.1 Discussion

There are no reports in the literature to compare the results of acid concentrations at different pH conditions. There is a study however, that reports the reduction in gluconic acid production with the increase in the acetic acid addition (Lu et al. 1999). The reason for the same was not confirmed whether it was due to acetic acid addition or the

subsequent lowering of pH. Their results are similar to those observed in the present study where the amount of gluconic acid is lesser when the acetic acid was added to the medium at pH 3.0 and 3.5 (Fig. 4.21). The acetic acid was added to the medium at the beginning of the runs controlled at pH 3.0 and 3.5 in order to lower the pH of the medium, which was usually observed to be approximately 4.5. In the runs controlled at pH 5.0 and 6.0 when acetic acid was not added but larger amount was produced by the bacteria, the gluconic acid produced was relatively less. The only exception was the runs controlled at pH 4 where acetic acid produced was less compared to the gluconic acid. Further studies also need to be carried out to confirm whether the acetic acid (external or produced by the cells) or the subsequent lowering of pH impacts gluconic acid production. It is interesting to note that the maximum gluconic acid was produced at pH 4.0 (Fig. 4.19 which is also reported as the pH of maximum oxygen uptake (Embuscado et al. 1994). This result indirectly indicates that maximum oxidation at pH 4.0 leads to the highest production of gluconic acid. Less acid was produced when the pH was not controlled. This could be due to less stress of pH change on the cells or the caustic in the current study for maintaining the pH could be having an influence on the biosynthetic activity of the cells. This could be a subject for future study. A study that conducted a glucose oxidase assay (GOD) reported that the GOD activity was dependent on the pH of the medium (Hwang et al. 1999). Their results indicated that the pH 4.0 was most favourable for the formation of gluconic acid from glucose and decreased with an increase in pH from 4.0 to 6.0. The study of Hwang et al. (1999) was in agreement with the results of the present study where maximum gluconic acid was estimated at pH 4.0 and lowest at pH 6.0. Further studies should be carried out to confirm whether GOD activity is the only factor that impacts the BC yield and production or one of the factors affecting the same.

4.16 Summary

In the present study, the rate of production of BC at $\approx 13 \text{ BC/m}^2 \cdot \text{day}$ in the static-cultures was about three fold greater than that observed in the rotating-bioreactor at $\approx 4 \text{ BC/m}^2 \cdot \text{day}$. This was contrary to the observations reported in published studies using the rotating-bioreactor (Serafica 1997). The yield observed in both the static-culture and the rotating-bioreactor was similar at $\approx 0.2 \text{ BC/g glu.}$ The approximate values have been calculated across the different varying conditions of culture conditions in both the static-culture and the rotating-bioreactor. The rate of production was varied, by controlling the tangential velocity of the rotating-bioreactor. Both the methods of BC production were influenced by the change in the glucose concentration and produced excessive acid at higher concentrations of glucose. This implies that glucose is preferentially converted into gluconic acid at higher glucose concentration while at lower concentration more biomass is formed. In the rotating-bioreactor the pH influenced both the yield and rate of production while acid was preferentially produced at pH 4.

CHAPTER 5

Properties of bacterial cellulose

5.1 Introduction

The physical properties of BC including WHC and tensile strength are a function of the growth conditions along with the structural morphology of the cellulose ribbons (Kato et al. 2007; Schramm and Hestrin 1954a). It has been observed in Chapter 4 that the change in conditions of growth influenced the morphology of the BC produced. In this chapter, the impact of the changes in structural morphology on important properties such as water holding capacity (WHC) and mechanical strength will be investigated.

5.2 Influence of the BC structure on the water holding capacity (WHC)

The most striking properties of BC include a very high water holding capacity (WHC) coupled with good mechanical strength in the never-dried state along with great elasticity and conformability (Czaja et al. 2005). The high WHC (approximately 200 times its dry mass) of wet BC is one of the most prominent and unique feature that has been utilized in many medical-based applications. According to the hypothetical model proposed by Colvin et al. (1977), the nascent chains of glucosan extruded by *G. xylinus* are attracted to each other by Van der Waals forces while being held apart by adsorbed water layers. This water layer forms a coating on the central sheath of the micro-fibril dictating its width. The removal of this water layer by methods such as air-drying or solvent exchange leads to the irreversible association of the hydroxyl groups of these chains to form crystalline BC microfibrils (Colvin and Leppard 1977). Hence, the WHC is calculated only in wet or never dried samples of BC. The water in the BC is entrapped within the fibrils hence the amount of WHC will depend on the dimensions and fibrillar arrangement. In Chapter 3, it was noted that there were differences not only in the microfibrillar dimensions but also in

the arrangement of the same depending on the conditions of production. These differences were very distinct in the BC produced by the different methods: the static-culture and using the rotating-bioreactor. The impact of these differences in structural morphology on the WHC has been investigated in the proceeding sections. The WHC was estimated using the method developed by Schrecker and Gostomski (2005) and the average sample size =20.

5.3 Influence of variation of medium conditions on WHC of BC produced in the static-culture

BC samples produced in different medium conditions of varying pH level and glucose concentration were macroscopically similar. No difference in the WHC was observed when these samples were handled manually. All the samples appeared similar and drained approximately equal amount of water on pressing. The samples were tough and required extra care in cutting them to size to avoid loss of water. The samples were stored in deionized water until tested to avoid loss of moisture due to evaporation.

The WHC was estimated for the varying initial glucose concentration and pH of the medium. The average WHC of the BC produced in static-culture at an initial glucose concentration of 50 g/L and an initial pH of 4.5 ± 0.5 was estimated as 93 ± 12.6 g_{water}/g_{BC} (mean \pm standard deviation with sample size=20). In the assays conducted at different initial glucose concentrations, the highest WHC was recorded at an initial glucose concentration of 2.2 g/L (Fig. 5.1). There was a significant difference between the WHC of BC produced at different initial glucose concentrations except between 11 and 46, 57, & 72 g/L. There was no significant difference between 21 and 87 between 39 & 57 and 72 g/L and also between 57 and 72 g/L (Appendix 3). There was no significant difference

in the WHC between the BC samples produced in static-culture at different initial pH statistically ($p < 0.05$ at C.I. = 95%) (Fig. 5.2) (Appendix 3).

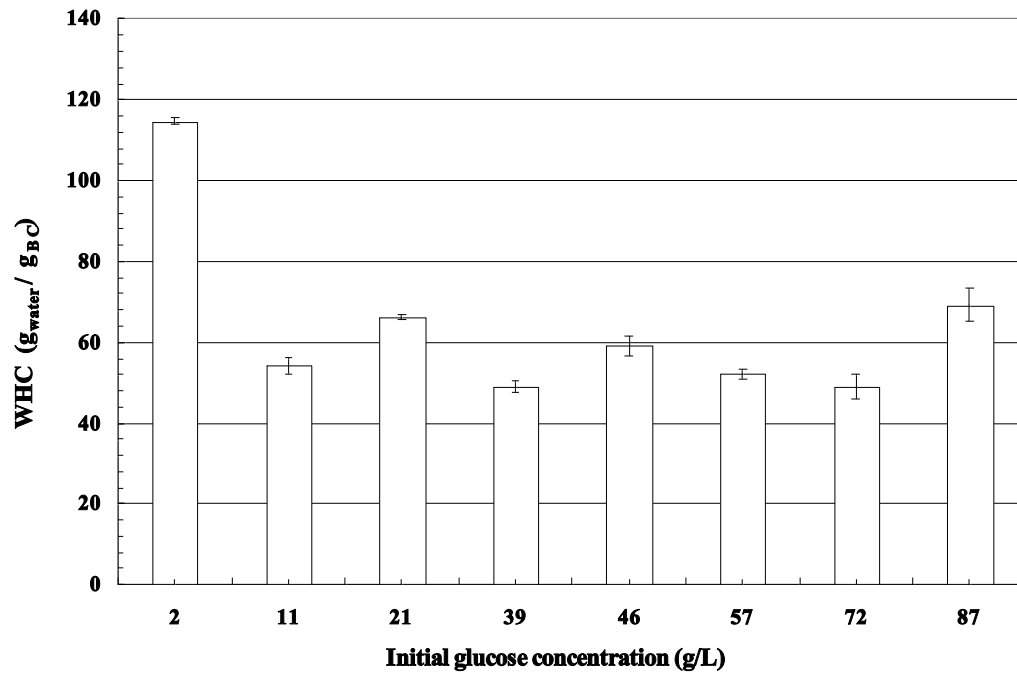


Fig. 5.1- The WHC of BC samples produced in the static-culture at different initial glucose concentration. The variation represents the difference in WHC of the samples. Sample size = 40, 5 samples (area = 490 mm²) from each of the 8 static-cultures for every glucose concentration.

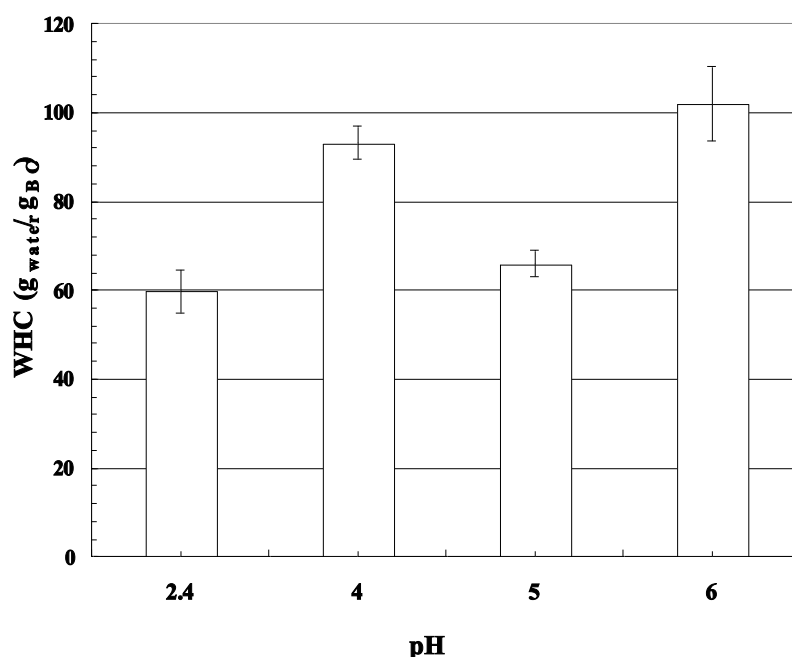


Fig. 5.2 – The average WHC of BC produced in static-culture at different initial pH and similar initial glucose concentration of 50 g/L. The variability represents the difference in the WHC within the samples at different pH (sample size=5 for each of the four different pH).

5.3.1 Discussion

While there are not many published reports comparing the WHC of BC produced in different medium conditions varying glucose and pH, there are some reports estimating the WHC produced in a static-culture. Seifert et al. (2004) reported the WHC of BC produced in the static-culture as 87% as compared to 92% produced in the static-culture in the present study. The small variability in the result could stem from the method and condition of medium and/ or the method used to test the WHC. They used the Jayme and Rothamel (1948) method for determining the WHC while the Schrecker and Gostomski (2005) method was used in the present study that is shown to have about 50% less variability compared to other methods. Another group reported the WHC at approximately 50 g water/g BC in the static-culture (Krystynowicz et al. 2002). The

difference in the result could again be due to the difference in the conditions of medium and/or due to the centrifuge method (Watanabe et al. 1998b) used for determination of WHC.

5.4 WHC of BC produced in the rotating-bioreactor

As mentioned earlier (Chapter 3, section 3.4) the main difference between the BC produced in the static-culture and the rotating-bioreactor macroscopically is the texture and the WHC. The BC samples produced in the rotating-bioreactor were relatively difficult to handle because of their very high WHC. The samples would easily drain water under gravity even when no force was applied. Therefore, extra care had to be taken while handling the samples specially when cutting the sample to the required size. Many samples were lost during this process. The macroscopic difference noticed in the samples produced at different pH (3.0-6.0) was characterized by the absence of macro-layers and were observed when pH was not controlled. These layers posed an additional challenge while cutting the samples. The samples produced at pH 3.0 and without active pH control (no pH control) appeared tougher and drained less water during handling compared to samples produced at other pH (3.5, 4.0, 5.0 and 6.0). The samples produced at higher tangential velocity (0.07 to 0.016 m/s) were the thickest (7 ± 3 cm) and drained more water while handling, while the samples produced at lower tangential velocities (0.01 to 0.06 m/s) were thinner (3 ± 2 cm) and drained lesser water during handling

5.5 Influence of the variation in pH on the WHC of BC produced in the rotating-bioreactor

In BC produced at different pH the WHC increased with the increase in pH from an average of 113 ± 8 g_{water}/g_{BC} at pH 3.0 to the highest average WHC of 162 ± 43 g_{water}/g_{BC} at pH 6.0 (Fig. 5.3). The lowest average WHC of 105 ± 6 g_{water}/g_{BC} was recorded in

samples produced without pH control (no pH control) (Fig. 5.2). Statistically, there was no significant difference between the WHC of BC produced at different pH except between some groups. The WHC at pH 6 was significantly different from WHC at pH 3.0, 3.5, no pH control & 4. Also the WHC of no pH control was significantly different from pH 5 and 6 (Appendix 3).

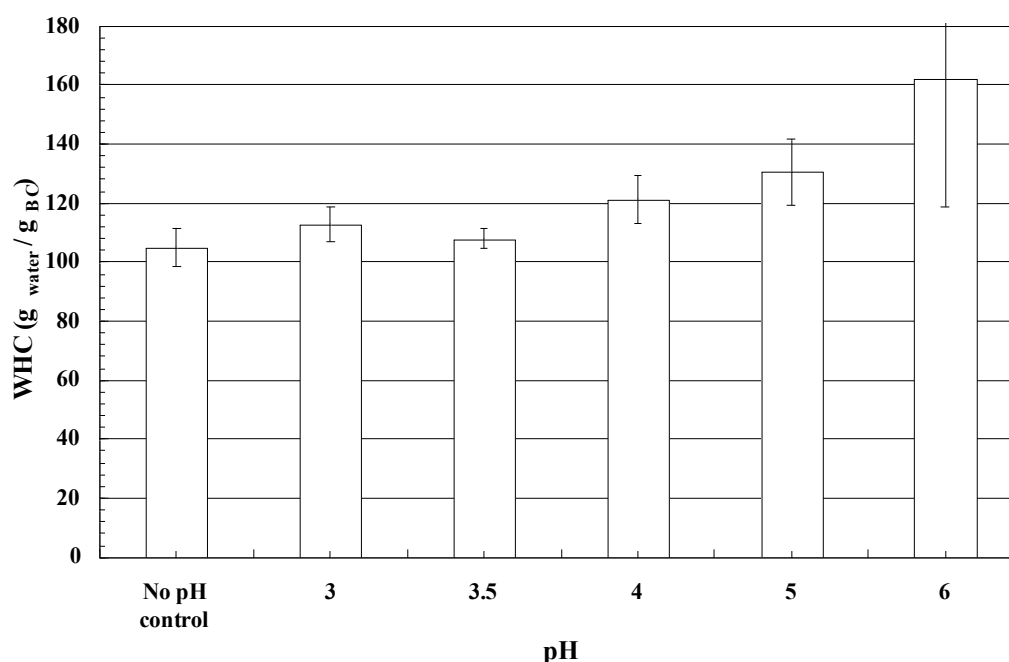


Fig. 5.3 -The average water holding capacity of BC produced at different pH levels in rotating-bioreactor at tangential velocity = 0.095 m/s and initial glucose concentration= 50 g/L. The variability represents the difference in the WHC between the BC samples (sample size= 20) from the two cylinders for each of the different pH levels.

5.5.1 Influence of the variation in the tangential velocity on the WHC of BC produced in the rotating-bioreactor

The WHC increased with an increase in the tangential velocity. The increase in the tangential velocity from 0.012-0.16 m/s corresponds to a non-linear increase in WHC from approximately 92 ± 9 to 176 ± 6 g water/g BC (Fig. 5.3). The average WHC of most

of the runs was between 100 and 120 g_{water}/g_{BC} (Fig. 5.3). The WHC was highest 176.3 g_{water}/g_{BC} at the highest tangential velocity of 0.16 m/s (Fig. 5.3). The WHC of the BC produced in the rotating-bioreactor at different tangential velocities was not significantly difference statistically between most groups ($p < 0.05$ at C.I=95%)(Appendix 3). There was significant difference in the WHC between 0.038 and 0.161, 0.138, 0.132 and 0.037 m/s. There was also significant difference in the WHC between 0.063 and 0.161, 0.138 m/s. Also there was a significant difference in WHC between 0.031 and 0.161, 0.138 m/s (Appendix 3).

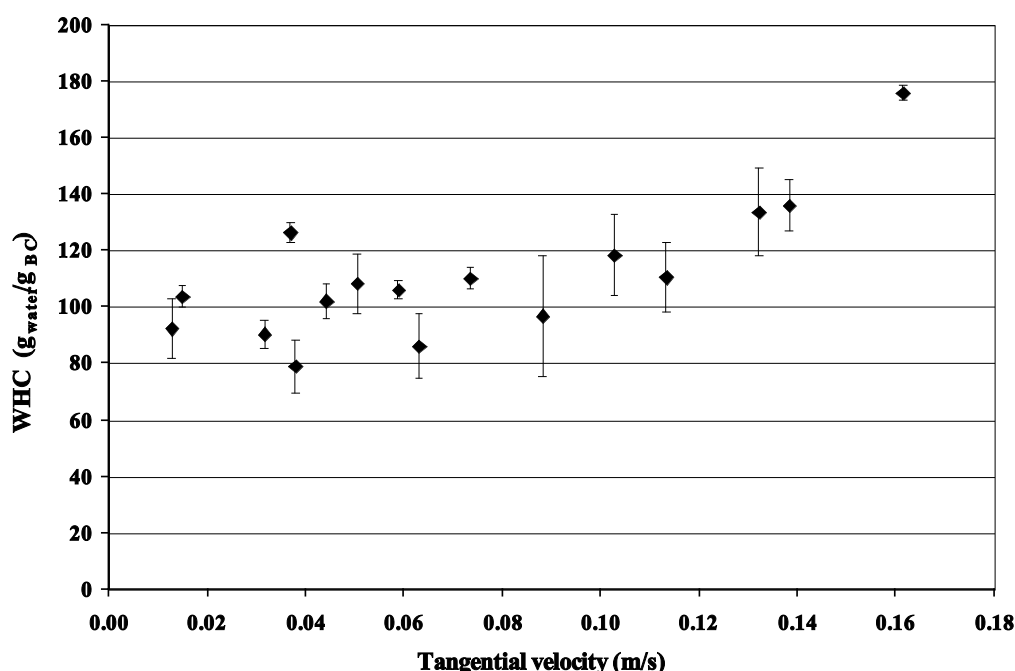


Fig. 5.4 - The average water holding capacity of BC produced at different tangential velocities. The variability represents the difference in the WHC between BC samples (sample size =10) from each the two cylinders for the different tangential velocities.

5.5.2 Discussion

There are very few published reports in literature about the WHC of BC produced in the static-culture and fewer reports on those produced in a rotating-bioreactor. There are no reports of the WHC of BC produced by different methods in varying growth conditions (pH and glucose concentration). The WHC of the samples produced at different pH were found to be statistically different ($p < 0.05$ at C.I. = 95%). The BC samples produced at lower pH of 3 and 3.5 showed lower average WHC of 113 ± 6 and 108 ± 3 g_{water}/g_{BC} respectively compared to the WHC of 130 ± 11 and 162 ± 43 g_{water}/g_{BC} observed in samples produced at pH 5.0 and 6.0 respectively. It has been reported that the water is held within the fibrillar structure (Colvin and Leppard 1977). Although the exact reason of observed higher WHC at higher pH (5-6) and vice versa is not confirmed. The structural difference between the samples produced at various pH values seems to be the probable cause for the variation in their WHC. It has been established (Chapter 3, section 3.7.2) that the cellulose network is comparatively more open at higher pH than at lower pH and the probable causes have been discussed. Since the fibrils hold the water between them by capillary forces, it is logical to presume that the more open structures with minimum scarring observed at higher pH (4.0, 5.0 and 6.0) hold more water compared to the BC produced at lower pH that has a tighter structure with excessive scarring.

The WHC at higher tangential velocities was greater than at lower tangential velocities. These results are in agreement with the higher hydration rates at increased rotations reported in the past (Krystynowicz et al. 2002; Serafica 1997). These groups did not offer any explanation for the same. It has been reported that nascent cellulose prior to crystallization holds more water than after (Kai and Koseki 1985). They speculated that the water molecules run through such cellulose in order to balance the intra and extra-cellular pressure. Thus, their function is to protect the cells at high pressures (100 MPa).

The pressure in the different runs was not altered but the increase in the tangential velocity can lead to increased shear stress (Serafica 1997). In order to overcome this stress the nascent cellulose may be harbouring extra water molecules leading to increased hydration at higher tangential velocities. Further studies need to be conducted to confirm this hypothesis.

Despite the variation in the pH and tangential velocities, the average WHC of the BC produced in the rotating-bioreactor at 115 g_{water}/g_{BC} was higher than in the BC produced in the static-culture at 92 g_{water}/g_{BC} (average WHC of BC produced in static cultures at sugar concentration of 50 g/L). This difference can again be linked to the structural difference between the BC produced in the different methods. The BC produced in static-culture method has a more closely knit network of ribbons with higher amount of scarring than the BC produced in the rotating-bioreactor.

5.6 Determining the WHC of BC produced in a rotating-bioreactor under tension

The WHC of the wet cellulose were determined by Schrecker and Gostomski (2005). In this method, the wet cellulose was subjected to a 98 Pa vacuum using a 1 cm hanging water column to remove the surface water for easier handling without draining water. In similar fashion, a series of assays were conducted under my supervision by Kim Langbein, an exchange student from Germany. In these assays, the wet BC produced in the rotating-bioreactor was subjected to varying vacuum conditions (post-production) by increasing the height of the hanging water column (0.5-75 cm). The equilibration time for the samples was maintained at four hours (Schrecker and Gostomski 2005) before recording the WHC. These samples were then subjected to rewetting for 24 hours either in single series or in two series where the BC samples were repeatedly drained and rewetted in two cycles and their WHC was recorded. All the BC samples used in the

assays were taken from a representative fermentation run (pH 4, tangential velocity 0.095 m/s, submersion level of 26% for the larger cylinder and 21% for smaller and the initial glucose concentration was 50g/L). These assays were not performed on samples produced in the static-culture.

Similar samples were subjected to different tension by varying the height of the water column height between 0.5, 1, 5, 25, 50 and 75 cm (Fig. 5.4). The WHC of the samples before and after rewetting for twelve hours was not significantly different in the first cycle ($p > 0.05$ at C.I=95%) (Fig. 5.4) (Appendix 3). However, there was a significant difference in the WHC of the samples under different tensions (0.5 to 75 cm). For example the difference between average WHC $156 \pm 20 \text{ g}_{\text{water}}/\text{g}_{\text{BC}}$ at the tension between 1- 5 cm was approximately eight times greater than the average WHC $17 \pm 0.2 \text{ g}_{\text{water}}/\text{g}_{\text{BC}}$ at the tension between 25-75cm (mean \pm standard deviation, sample size=5 for each tension). The one-way analysis of variance (ANOVA) suggests two groups based on the different tension: one group includes samples under tension between 0.5 to 5 cm and the other 25-75cm(Appendix 3). The WHC between the two afore mentioned groups was not significantly different at the tension of 25, 50 and 75cm and the tension of 0.5, 1 and 5 cm ($p > 0.05$ at C.I=95%) (Fig. 5.4)(Appendix 3). These samples were further rewetted for 24 hours and the WHC was determined for samples at an equilibration of four hours each at similar tensions they were before subjected to rewetting (Fig. 5.5). It was found that there was no significant difference between the WHC of the samples before and after rewetting for 12 hours ($p > 0.05$ at C.I=95% (Fig. 5.4). After conducting WHC assays with one rewetting cycle, it was decided to test whether two rewetting cycles would influence the WHC. In order to test the same a representative sample were chosen and drained for four hours under tension of 1 cm. The choice of this tension is

based on the fact that 1 cm tension was used to estimate the WHC throughout the present study.

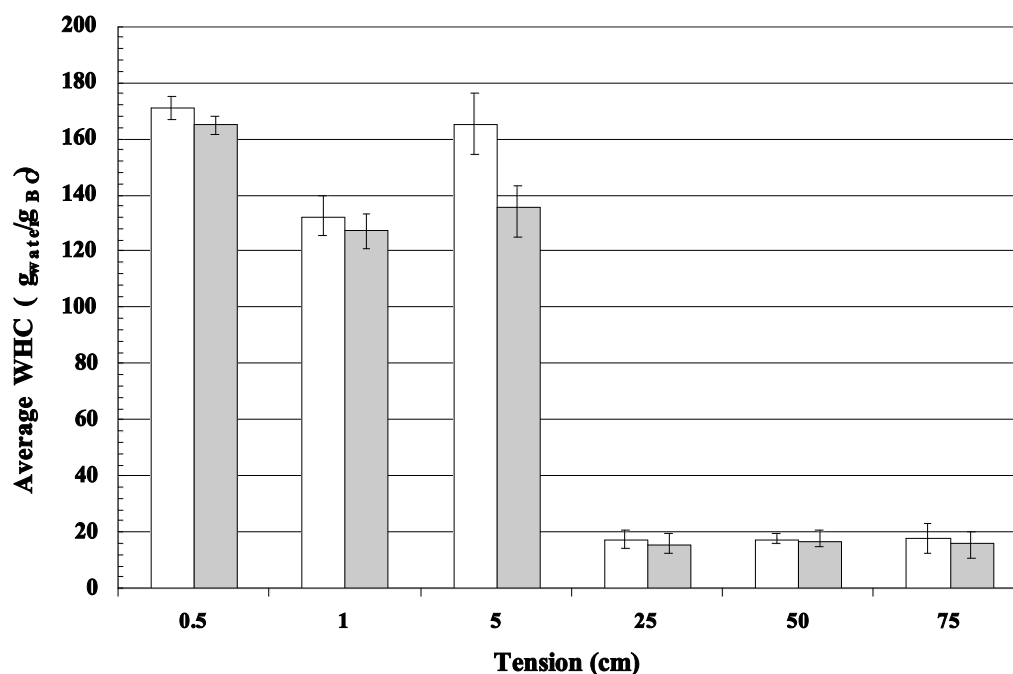


Fig. 5.5- The average WHC of wet BC produced in the rotating-bioreactor under tension at different heights of water column (0.5, 1, 5, 25, 50 and 75 cm) (white bars). The average WHC of the same samples subjected to similar tensions after rewetting in water for 12 hours (grey bars). The equilibration time of four hours was maintained for each sample. The variability represents the difference in the WHC between the samples at different tension (sample size = 5 each for different tension).

5.6.1 WHC of BC samples rewetted in two cycles

The WHC of the BC samples were estimated under 1 cm tension, the samples were then rewetted in water for 12 hours and the WHC was re-estimated at 1 cm tension followed rewetting in water for another 12 hours thereby completing two rewetting cycles. Thus the samples were subjected three cycles of determining WHC using 1 cm water column and four hours of equilibration time and two cycles of rewetting. There was a decrease in

the WHC from an average of 159 ± 4 to 136 ± 11 g_{water}/g_{BC} in the first cycle and in the second cycle got further reduced although negligibly to 132 ± 9 g_{water}/g_{BC} (mean \pm standard deviation, sample size=5 for each tension) (Fig. 5.6). It was found that the average WHC of the samples drained for 4 hours and subjected to one cycle and rewetting for 12 hours was not significantly different from the WHC of the samples before rewetting ($p>0.05$ at C.I.=95%) (Appendix 3). There was significant difference between the average WHC of samples not subjected to rewetting cycle and those subjected to two rewetting cycle ($p<0.05$ at C.I = 95%) (Appendix 3).

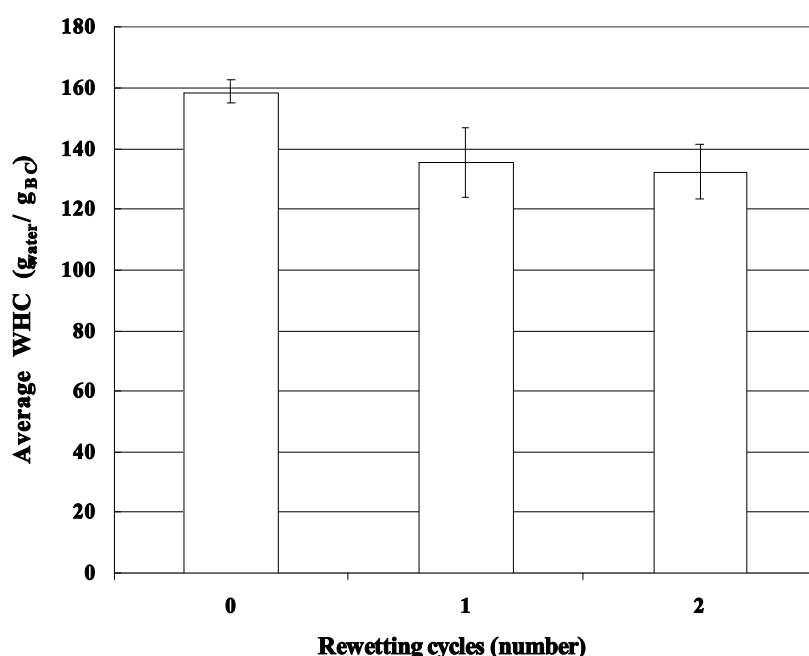


Fig. 5.6 - The average WHC at tension= 1cm for 4 hours and two cycles of wet BC samples produced in the rotating-bioreactor. The samples were rewetted in water for 12 hours and the WHC was recorded at tension = 1cm (cycle 1). The same samples re-immersed in water for 12 hours for the second time and the WHC was recorded at tension= 1cm (cycle 2). The variability represents the difference in the WHC between the samples (sample size=5).

5.6.2 WHC of air-dried BC samples produced in the rotating-bioreactor

The WHC of assays were carried out after rewetting air-dried BC samples produced in the rotating-bioreactor at different tangential velocity, pH and initial glucose concentrations. The average WHC of these samples was 13.5 ± 4.1 g_{water}/g_{BC} (mean \pm standard deviation, sample size= 5 for each tension). The average WHC of air-dried rewetted BC samples produced in static-culture was 10.32 ± 3.6 g_{water}/g_{BC} (mean \pm standard deviation, sample size= 20). Despite rewetting the air-dried samples in water for different duration of time there was not much change in the WHC. A representative sample was taken from BC produced in rotating-bioreactor at pH 4 at an initial glucose concentration of 50 g/L and tangential velocity of 0.095 m/s. The air-dried BC samples were immersed in DIW for different length of time (24, 48 and 96 hours). The WHC of the samples was estimated similar to the wet (never dried) samples using the vacuum method (Schrecker and Gostomski 2005). The average WHC of the air-dried BC samples rewetted in water for different duration of time was similar (Fig. 5.6). The WHC of the rewetted air-dried samples for different duration of time was not significantly different statistically ($p>0.05$ at C.I= 95) (Appendix 3).

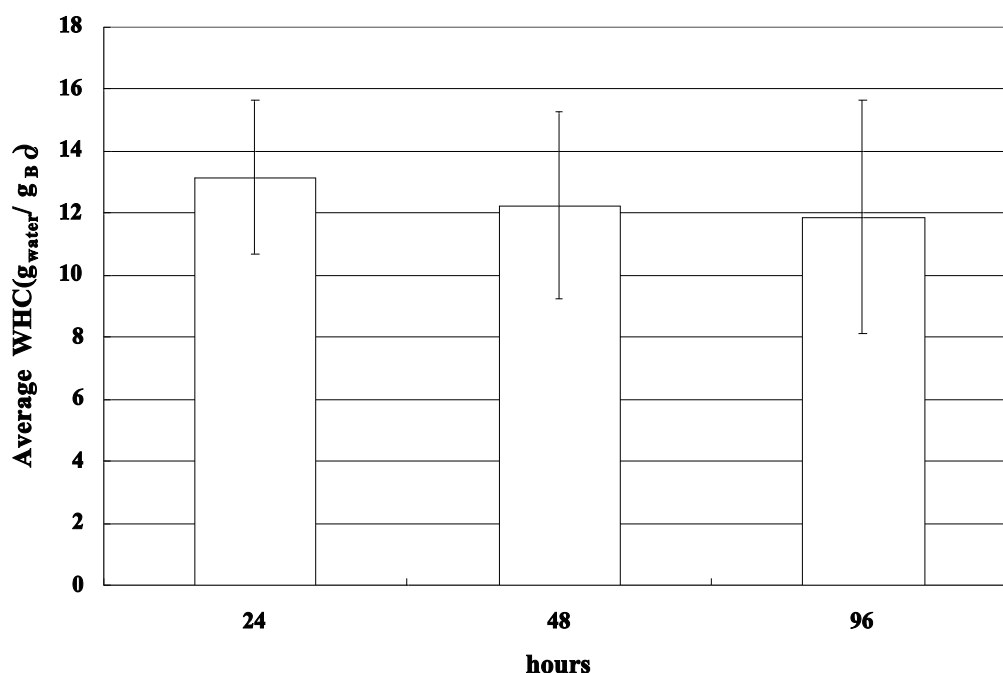


Fig. 5.7 - The WHC of air-dried BC samples produced in the rotating-bioreactor immersed in DIW for different length of time (24, 48 and 96 hours). The WHC was determined using the vacuum method and sample size=10.

5.6.2.1 Discussion

When the wet BC samples produced in the rotating-bioreactor at pH 4 were drained for four hours at a maximum tension of 1 cm (98 Pa under vacuum) the WHC was not impacted. This was confirmed by rewetting the samples in water for 12 hours before re-calculating their WHC (Fig. 5.5). This result support the hypothesis of Schrecker and Gostomski (2005) who reported the minimum tension for draining the wet BC samples as 1 cm and duration four hours. However, when the samples were under similar tension for the second time the WHC reduced (Fig. 5.5). This observation suggests a change in the fibrillar structure of the BC when subjected to a tension of 1 cm the second time. This change could be due to the increase in inter-fibrillar bonding although it has not been confirmed. While this change has not been studied in the current study, it has been

investigated in the past by Schrecker (2004). He reported a change in the structure, as the ribbons melted into one another also known as scarring with the increase in tension from 49 Pa vacuum to 196 Pa and finally to 384 Pa (Fig. 5.7). The increase in the vacuum led to an increase in the scarring until the BC began to look almost sheet-like. This explains the reduction in the WHC with the increase in tension in the present study. In the current study much greater tension was applied, for example 2450 Pa at a water column height of 25 cm, that could probably explain the fall in WHC by eight times. Also, the structural change was permanent hence despite immersion in water for 12 hours there was no increase in the WHC.

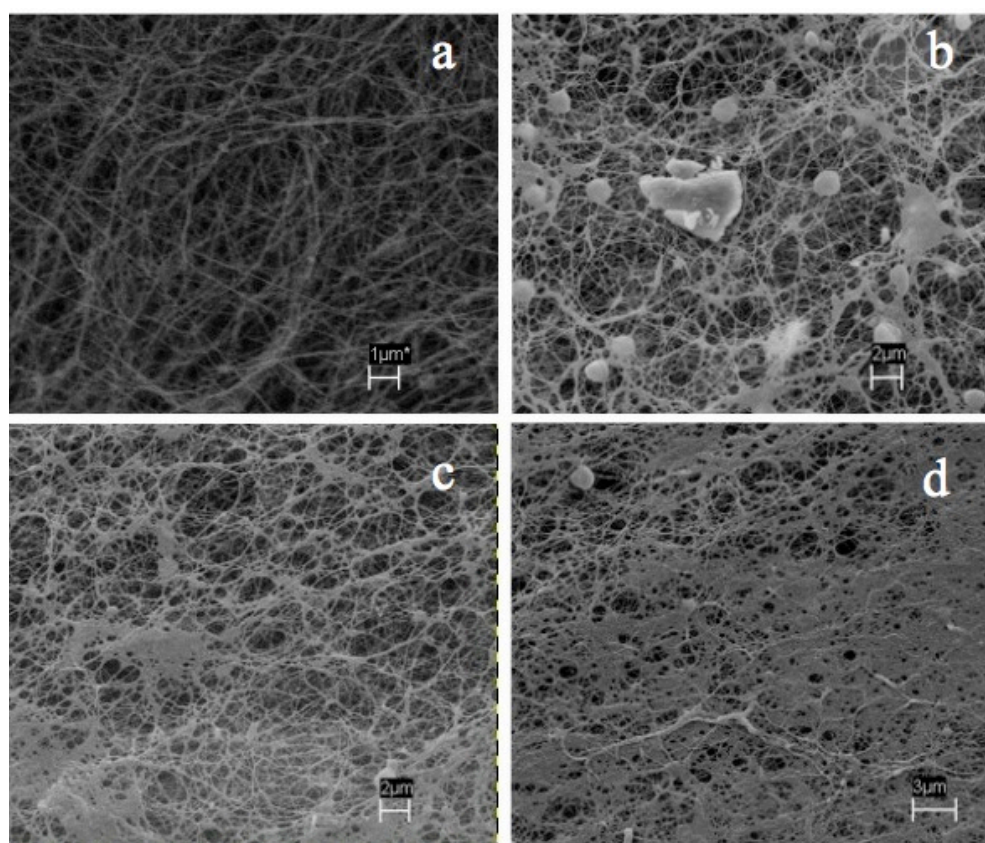


Fig. 5.8 - SEM micrographs of freeze-dried samples of BC produced in to rotating-bioreactor subjected to different levels of tension (Fig. a=0, b=49, c=196 and d=384 Pa) in the wet state. Adapted from Schrecker (2004)

Similar observations were made in air-dried samples immersed in water (Appendix 3). The statistical analysis ($p > 0.5$ at C.I = 95%) showed no significant difference in the WHC of dry samples immersed in water for any length of time (24, 48 or 96 hours) (Appendix 3). There was a reduction in the WHC by $\approx 90\%$ and no change in the WHC despite immersion of the dry BC samples in water from 24-96 hours. This drop in hydrophilicity of the dry BC samples can be attributed to the strong hydrogen bonding due to loss of water during drying which resist rupture by rewetting (Westman and Lindström 1981).

In future, these assays should also be conducted on BC samples produced in static-culture. This would help us know the difference in their draining ability of BC produced by different methods. BC samples of different thickness too should be tested to investigate impact of tension on varying thickness. These assays are important from an application point of view. BC is used increasingly in the medical field specially as wound dressing (Klemm et al. 2001), this assays could give an indication as to the amount of exudates the wound dressing can hold in the wet state. These assays can also be indicative of the amount of tension they can withstand before a permanent change in the structure and the subsequent WHC. There is no published literature on these assays for comparison. In the current study a crude set-up was used which can be further refined for future use.

5.7. Mechanical properties of BC produced in static-culture and the rotating-bioreactor

In the previous chapter it has been established that the ribbon width and their arrangement varies with respect to the method of production (the static-culture or the rotating-bioreactor) and variation in the conditions of growth (pH) within the methods. This difference in the ribbon structure could have an impact on the mechanical properties of

the BC (Zhou et al. 2007b). The details of these investigations are discussed in the following sections.

5.8 Background research

The mechanical testing of wet (never-dried) bacterial cellulose (BC) was a challenge owing to the nature of the material. There have been reports in literature of mechanical testing of wet BC but most of them had been produced by the static culture method wherein the material had a higher mechanical strength hence easier to handle. The BC produced using the rotating-bioreactor had lower mechanical strength coupled with very high WHC and made mechanical testing more difficult. The popular approach of extending the BC samples under stress to failure using testing device such as Instron and Dynamic mechanical analyser (DMA) was tried without much success.

BC was difficult material to work with because of its slippery texture. It was difficult to hold the BC within the grips of any mechanical testing apparatus due to its smooth and wet texture. There was a bigger difficulty in inserting the hydrated slippery samples into the grips without damaging the structure. Even if the sample were gripped well enough, the material tended to fail at the grips, which invalidates the test. The other difficulty was that some of the BC samples were layered and the layers would slip during testing giving a false reading. To overcome the problems mentioned and on the suggestion of Dr. Nick Tucker (CFRI, Christchurch) small tabs of Scotch Brite (household scrubber) material were sewed to the felt on which the BC is formed so that the BC would grow into the Scotch Brite tabs and could be used to grip the samples (between the scotch brite tabs). When this method was tried using the DMA it was easy to hold the rigid tabs but the sample in between them was not sufficiently rigid. When one tab was held in the upper clamp, the weight of the lower tab had a deteriorating impact on the BC. Hence, this

technique was abandoned as it required further development. Dr. Roger Newman (Scion, Rotorua), suggested the use of bloom strength test due to the gel-like nature of BC. But on discussion with Dr. Kathleen Hoffman from (CFRI, Nelson) who was experienced in conducting the bloom strength test, she advised that maintaining the concentration of the jelly was very critical for effective testing. In case of BC, it is difficult to maintain the concentration of the material thereby affecting the accuracy of the bloom strength test.

A series of tensile tests were conducted on fully hydrated BC using different mechanical testing apparatus including Material Testing System (MTS), the Instron and Dynamic mechanical analyzer (DMA) without much success. After facing a number of problems while conducting the tensile tests with wet BC, advice from Dr. Nick Tucker (Crop and Food Research Institute, Christchurch), and Dr. Roger Newman (SCION, Rotorua) led to the conclusion that the fully hydrated BC material was hydrogel in nature. The fully hydrated BC satisfies the criteria of hydrogel that is a polymer made up of a network of monomers held together by bonds capable of absorbing 20-1000% of their dry weight in water. After a final round of discussion with Dr. Susan James, a visiting professor of biomedical engineering from the Colorado State University (USA) who was experienced in conducting mechanical testing on hydrogels, it was decided to use the compression method for testing the mechanical strength of wet BC. The Dynamic mechanical analyzer (DMA) was the first instrument of choice for this test because it has a lower load cell of 1kg that would give more accurate result. However, there was a fault in the operative software of the DMA (Department of Mechanical Engineering, University of Canterbury, and Christchurch). It was found that that in the compression mode, the platen in the DMA could travel only 5 mm downwards. This was not sufficient for BC samples in the current study as they ranged between 1 to 10 mm in thicknesses (Staiger 2008). Finally, it was decided to use the Instron Testing Machine UTM (model 1011) at the CFRI,

Christchurch, which had a higher load cell 5 kg but could compress thicker samples compared to the DMA.

5.9 Compression tests

The mechanical strength of different types of gels (Svensson et al. 2005) and biofilms (Körstgens et al. 2001) was investigated in the past using the compression test. In this test, the material was compressed between two plates (made of some solid material). The amount of force applied to the surface of the material and the distance travelled to the point of failure of the structure under compression was used to calculate the stress and strain values that can be translated into the mechanical strength of the material (Ahearne et al. 2008). The advantage of this method was that the geometry of the hydrogel was not limited and these tests can be easily set up and conducted under submerged conditions. Although, this technique had limitations including bulging of the material under compression and the difficulty of uniform application of force (Ahearne et al. 2008).

The compression tests were carried out in a specially built rig (Material and Methods Chapter 2, Fig. 2.4). The samples were placed in confinement to overcome the probable bulging problem. The bottom of the platen used for compression was a porous, sintered bronze material to allow water to flow freely through the platen into the water chamber without exerting an additional force on the material. The compression tests were conducted under water in order to eliminate the drying /draining of the BC samples that impacts the structure and mechanical strength. The platen was sonicated before the tests to remove the air from the pores and to avoid air re-entry, it was submerged under water throughout the compression tests.

The BC samples were placed in the confinement under water and compressed with the help of a platen that was positioned at a distance of about 25% of the initial thickness of

the sample. During compression, the force applied over the total distance until rupture (just after the maximum force was attained) was recorded. The speed of compression was 0.1 μm per second. The stress σ or force causing deformation was calculated by relating the applied force f to the initial cross-sectional area of the sample A_0 as described in the following (Eq. 1) (Körstgens et al. 2001).

$$\sigma = f / A_0 \quad (\text{Eq. 1})$$

The average initial film thickness d_0 was measured with the help of vernier callipers and scale. The actual distance travelled during compression d was used to calculate the difference Δd (Eq. 2) (Körstgens et al. 2001). To calculate the strain ε or the ratio of change (deformation) caused by stress (Eq. 3) (Körstgens et al. 2001).

$$\Delta d = d - d_0 \quad \text{where } d \leq d_0 \quad (\text{Eq. 2})$$

$$\varepsilon = \Delta d / d_0 \quad \text{where } \varepsilon \leq 0 \quad (\text{Eq. 3})$$

The values of stress and strain in compression assays are negative by definition and have been converted before reporting.

A stress strain curve was plotted using the values derived from Eq. 1 and Eq. 3. A typical compressive force - deformation is presented in Fig. 5.8. This curve can be divided into three stages. The stage 1 represents the force applied to the BC samples while the water was displaced. The stage 2 which is a fairly linear part of the curve resembles a Hookean solid. The extrapolation of this linear region gives the value Δd , which is the actual distance travelled from the surface of the sample until the point of rupture from the d_0 as calculated from Eq. 2. These values are then used to calculate the strain ε using Eq. 3. The forces measured throughout the linear part of the curve in stage 2 are used to calculate the stress developed in response to the strain using the Eq.1. The stage 3 occurs

when the linear curve ends indicating rupture of the structure as no further force is developed.

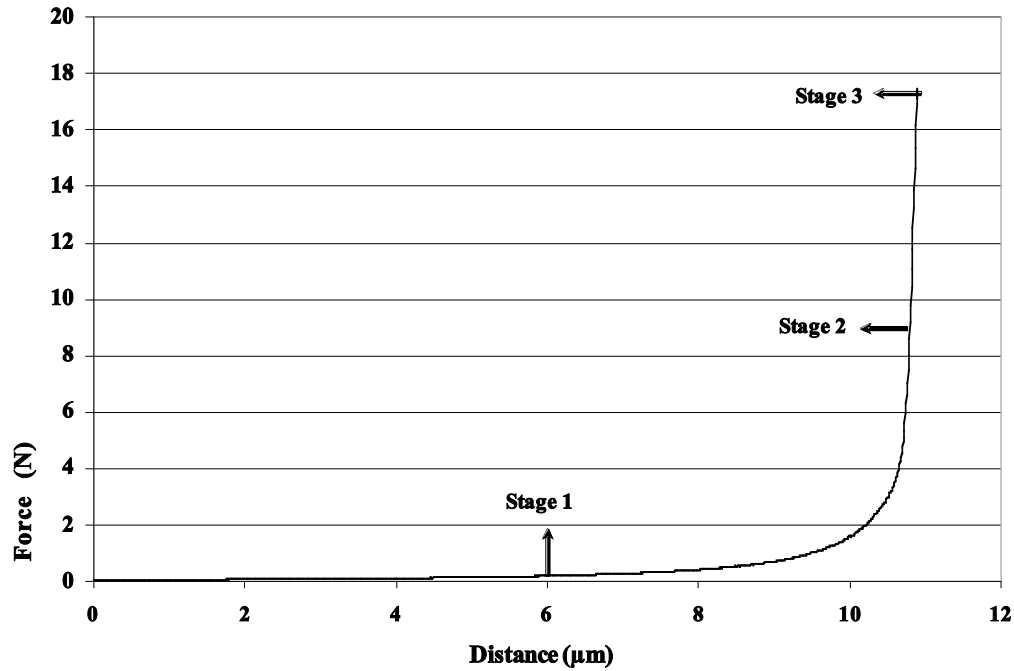


Fig. 5.9 - A typical force- displacement curve for fully hydrated BC at a constant displacement speed of 0.1 μm per second.

Once the strain and the corresponding stress were calculated using the Eq. 1 and Eq. 3, the points were plotted in order to get a stress vs. strain curve that was used to calculate the modulus of elasticity (MOE), or compressive modulus (CM) as referred to in the current study commonly calculated by the Eq. 4 (Körstgens et al. 2001). It is the slope of the line drawn from the linear portion of the stress/ strain curve (stage 2 in Fig.5.9).

$$E = \sigma / \varepsilon \quad (\text{Eq. 4})$$

A typical stress / strain curve is represented in the Fig. 5.9 and is similar to the curve obtained during the compression of BC in a previous study (Bodin et al. 2007b). The CM was calculated from the slope of the linear part of the stress/strain curve. The maximum stress develops at rupture of the structure and is usually referred to as ultimate tensile

stress (UTS) since this test is under compression it has been denoted as ultimate compressive stress (UCS) (Fig. 5.9).

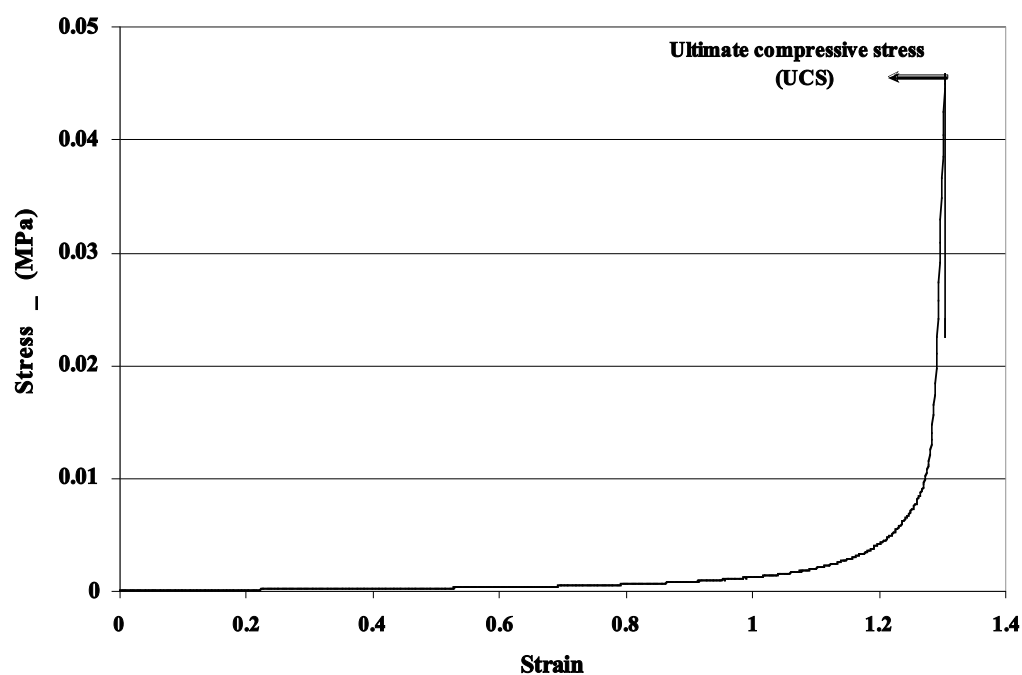


Fig. 5.10 - The typical stress/strain curve obtained for the entire compression test conducted.

In stiff materials like metal, the compression occurs in only one direction over a constant volume of the sample. It is very important to ensure lateral extensions to get meaningful data over the range of strain in uniaxial compression. But in samples such as BC that resemble a hydrogel, the Young's modulus described here as the compressive modulus (CM), depended on the thickness (Ahearne et al. 2008). The sample thickness varied between runs from 3 to 10 cm. Therefore, the strain was normalized for the sample thickness.

There was a difference in the strength of the samples tested based on the orientation of the sample. The samples compressed perpendicular to the direction of growth or layers had very small values of compressive modulus and could not be used for comparative

analysis. Whereas, the samples compressed parallel to the direction of growth had relatively higher compressive modulus and was used for mechanical analysis.

Samples of BC produced in the static-culture were also tested for comparative analysis and there was no significant difference in the compressive modulus of BC produced in static-culture at different initial glucose concentration and initial pH. Similarly, there was no significant difference between the CM of the BC produced at different initial glucose concentration in the rotating-bioreactor. Please note that compression tests samples produced at tangential velocity 0.013 and 0.015 m/s were not performed because of loss of samples due to damage.

5.10 The influence of the change in tangential velocity on the mechanical strength of the BC

In order to test whether the change in tangential velocity had an impact on the mechanical properties of the BC produced in the rotating-bioreactor, samples (sample size=10 for each tangential velocity). The samples were taken from both big and small cylinders as they both had different tangential velocities, which gave a wider range of data points. The average compressive modulus (CM) was 0.044 MPa. The highest compressive modulus of 0.076 MPa at a tangential velocity of 0.1 m/s and was the lowest at 0.02 MPa at 0.138 m/s (Fig. 5.11). Statistically, there was no significant difference between the CM of the BC produced in the rotating-bioreactor at different tangential velocities ($p>0.05$ at C.I=95%) except between some tangential velocities (Appendix 3). The CM at the tangential velocity 0.103 m/s was significantly different from the CM of all the tangential velocities (0.31, 0.044, 0.063, 0.073, 0.88, 0.113 and 0.132 m/s) (Appendix 3). A trend emerged as the CM increased non-linearly from 0.03 to 0.076 MPa with an increase in

tangential velocity from 0.03 to 0.1m/s. Further increase in tangential velocity to 0.16 m/s led to a reduction in the CM to 0.023 MPa.

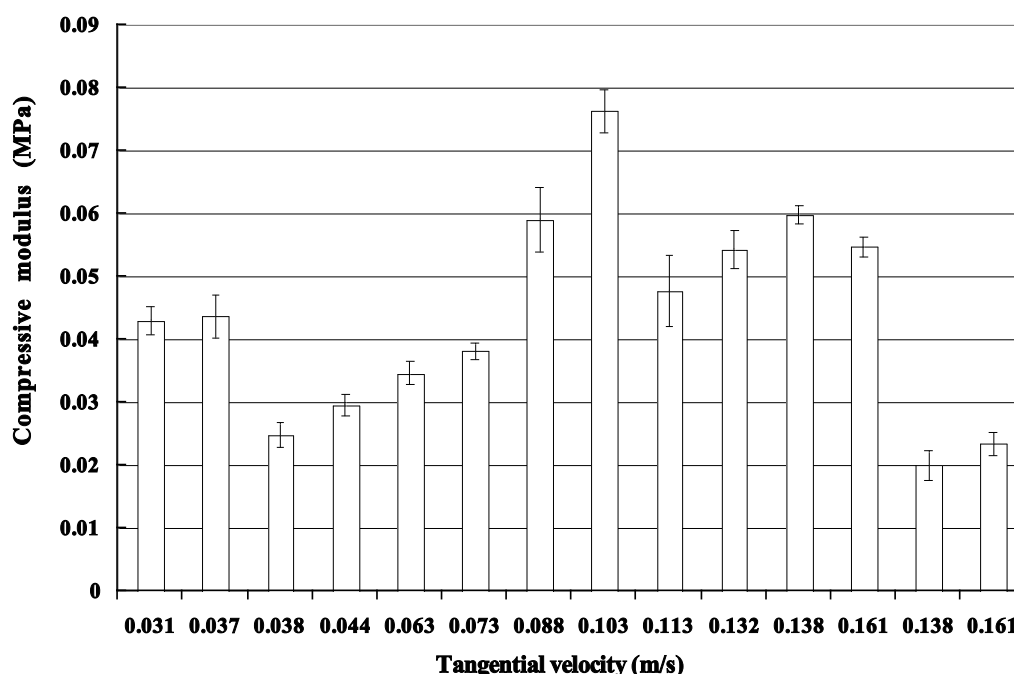


Fig. 5.11 - The compressive modulus of BC samples from both the cylinders runs at different tangential velocities (pH 4.0). The error bar shows the variability of the compressive modulus within the different samples at various tangential velocities (sample size=10 for each tangential velocity).

5.11 Influence of pH on the mechanical properties of BC produced in a rotating-bioreactor

The mechanical strength of BC produced at different pH was tested under compression. It was found that the BC produced with no pH control had higher mechanical properties with CM of 0.08 ± 0.003 MPa followed by BC produced at pH 4.0 at 0.067 ± 0.006 MPa (mean \pm standard deviation, sample size=10) (Fig. 5.11). The BC samples produced in pH 6.0 had the lowest CM at 0.003 ± 0.0015 MPa (mean \pm standard deviation, sample

size=10). None of samples produced in the rotating-bioreactor at any pH level were comparable to the samples produced in static-culture (initial glucose concentration = 50g/L) with the average CM of 0.46 ± 0.06 MPa (standard deviation, at samples size=10) (Fig. 5.12). Statistically, there was significant difference in the CM of the BC produced in the rotating-bioreactor at different pH except in certain groups ($p < 0.05$ at C.I. =95%) (Appendix 3). There was no significant difference between the CM of the BC produced at pH 3 and 6, pH 4 and 5, pH 4 and no pH control (Appendix 3).

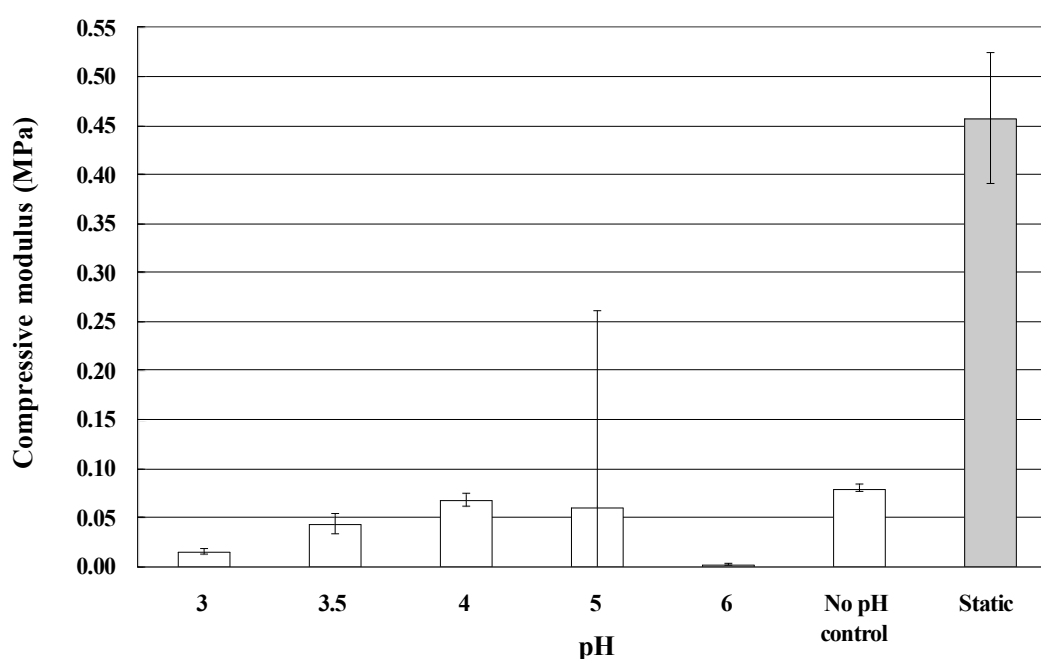


Fig. 5.12- The Compressive modulus of BC produced in the rotating-bioreactor at different pH (white bars) (tangential velocity = 0.095 m/s) and in a static-culture (grey bar). The error bars represent the variation between the samples from the same pellicle and run (sample size 10).

5.11.1 Discussion

Nakayama et al.(2004) reported that in compression testing, selecting the side which has to be compressed is very important. They tested BC samples produced in static-culture

under compression and found that the samples had a greater mechanical strength (2.9 MPa) when compressed on the layered or growing side of the pellicle, as compared to the side perpendicular to the layering end (0.007 MPa) which was about 400 times less than that of the layered end. Similar observations were made in the current study whilst conducting the compression tests using samples from both the static-culture and the rotating-bioreactor. The mechanical test conducted on BC perpendicular to the growing end in contrast to the parallel side was about 50 times smaller in BC produced in rotating-bioreactor as compared to the BC produced in static-culture under similar conditions of test. Therefore, all the compression tests were conducted on the layered or growing side of the pellicle. The buoyancy effects were corrected by deducting the blank measurement (the force exerted on DIW with the probe) from each of the compression test (Ferrari et al. 1995). The compressive modulus of BC samples produced in a static-culture was reportedly ranged from 0.0018 to 0.0035 MPa (Bodin et al. 2007a). This compressive modulus of the samples produced in the static-culture was much lower than that estimated in the current study at 0.46 MPa. The compressive modulus was even lesser than average of 0.045 MPa of the samples produced at different pH in the rotating-bioreactor in the current study. This difference may have been the result of various reasons including difference in strain and the method of testing.

Putra et. al (2008b) produced tubular BC using the static-culture method and under tensile testing determined the MOE of wet (never-dried) samples from the static-culture between 0.02 - 0.06 MPa breadth wise and lengthwise respectively. This was lesser than compressive modulus of the static-culture samples in the current study, but comparable to the samples from the rotating-bioreactor. This difference could be due to the difference in the method of testing. However, the ultimate fracture stress was in a range of 0.37 to 0.59 MPa breadthwise and lengthwise, respectively, and is comparable to the ultimate

compressive stress reported in the current study. The greater tensile strength of the tubular BC lengthwise as compared to breadth wise was attributed to impact of the curvature of the tube and the orientation of the microfibrils (Putra et al. 2008b). They further observed that the orientation of the microfibrils was affected only by the inner diameter of the silicone tube and was independent of the internal morphology of the tube. Since the BC was produced externally on circular shaped cylinders the study of the fibrillar orientation and their tensile strength on cylinders of different dimensions would make an interesting study.

The mechanical properties of the hydrogel depend on the amount of water present in it. The modulus of elasticity (MOE) and the ultimate compressive stress (UCS) decrease with a increase in water (Nakayama et al. 2004) supported by the power-law relation. This can probably explain the decrease in CM with the increase in tangential velocity beyond 0.1 m/s, which corresponds to increase in the WHC (Fig. 5.11). Another study reports that the rate of cellulose production is inversely proportional to the density of the cellulose ribbon network in the static-culture (Watanabe and Yamanaka 1995). They observed that with the increase in rate of BC production, BC fibrils of shorter branches were produced. This reduced the density of the pellicle produced and subsequently compromised its strength. The hypothesis of higher rate of production produces less dense BC can be applied to the BC produced in the rotating-bioreactor in current study. This was based on the observation that the increase in the rate of production was followed by a decline in the yield (Fig. 4.15 and 4.16). Hence a reduction in the density of the BC (as extrapolated from lesser yield) was observed when the tangential velocity was increased from 0.01 to 0.16 m/s. Interestingly, the comparison of compressive modulus (Fig. 5.11) at varying tangential velocity complements the yield (Fig. 4.16) at similar

tangential velocity. This implies that the rate of production impacts the yield which further influences the strength of the BC produced.

There is a paucity of published studies on the mechanical properties of never-dried or wet BC and even lesser of those produced in the rotating-bioreactor. Most of the published literature is based on the BC produced in the static-culture. The reason for difference in the mechanical strength reported in the various published studies could be caused by the fact that no two studies used a similar strain of *G. xylinus*. According to Clasen et al.(2006) the mechanical strength of the BC varies with the bacterial strain used. The cylindrical shape of the probe used in these compression tests is not the most appropriate, as another study suggests that among the three shapes tested (cylindrical, conical and spherical), the cylindrical probe (similar to the one used in current study) is not best suited for quantitative mechanical analysis (Ferrari et al. 1995). This was due to the complexity of the deformation which includes a combination of shear forces at the flat edges and compression at the centre. The compression tests conducted in the current study does not give a complete biomechanical picture of the BC produced under different conditions. Additional method development for testing the tensile strength of the material is required.

5.12 Qualitative analysis of mechanical strength in comparison to compression testing

Although the compression testing indicated that the BC produced at pH 5 had a greater CM than the BC produced at pH 6, qualitatively (by pulling the BC apart manually) it was found that the BC produced at pH 6 had higher tensile strength than that produced at pH 5. However, qualitative tensile testing of the static culture BC matched the CM testing indicating it was stronger material. Further research is required to quantitatively test the

wet BC under tensile conditions before arriving at any conclusion with regards to the mechanical strength. The only clear conclusion is that the BC produced in the static-culture had greater mechanical strength than that produced in the rotating-bioreactor. This was confirmed under compression testing and also qualitatively.

5.13 Tensile testing of dry BC samples

Tensile tests performed using wet BC samples from both the rotating-bioreactor and the static-culture were performed without much success. Therefore, tensile tests were performed on oven-dried BC samples from both methods (the static-culture and rotating-bioreactor) for comparative purposes. The focus of the current study was to use BC produced in the rotating-bioreactor in the wet form.

Mechanical testing was carried out on dry samples using the Mechanical testing system (MTS) (Chapter 2, section 2.16.8.1) . Only observations from samples that failed at the centre of the two grips were recorded. The data was recorded in the form of load (N) versus displacement (mm) for all the samples. This data was then converted into stress and strain. This was done by using the sample thickness and the initial gauge length (the length of the sample held between the two grips before the test). The thickness of the sample was measured using a micrometer. The calculation of the stress and strain values was similar to that in the compression test (Fig. 5.9).

The stress was calculated by the equation (1)(Askeland 1996).

$$\sigma = F / A_0 \quad (\text{Eq. 1})$$

Where σ is the stress, F is the force (Newtons) and A_0 is the original area of cross-section of the sample (mm^2).

The strain was calculated by the equation(2)(Askeland 1996).

$$\varepsilon = (L - L_0) / L_0 \quad (\text{Eq. 2})$$

Where ε is the strain, L (mm) is the final extension and L_0 (mm) is the gauge length.

The modulus of elasticity (MOE) was extrapolated from the slope of stress versus strain curve (Fig. 5.13).

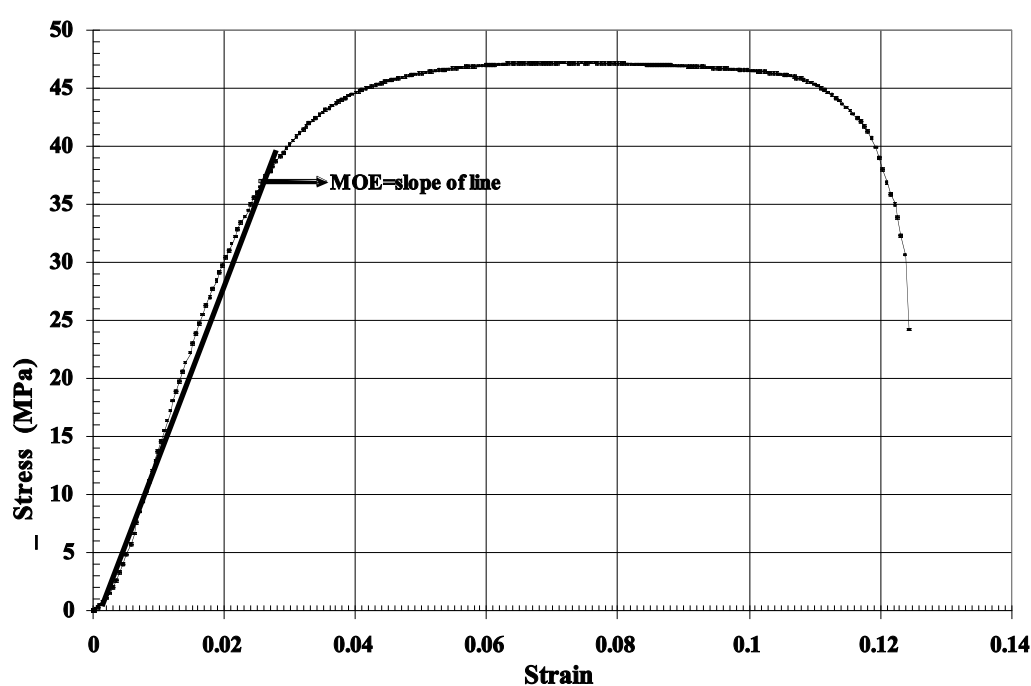


Fig. 5.13 – The graphical representation of: the calculation for the modulus of elasticity (MOE). The slope of the line on the linear portion of the stress/strain curve was used to calculate the MOE of dry BC samples.

There was a difficulty in conducting these tests with dry samples because most of them failed at the sites of grip and very few samples failed at the centre of the samples between the grips. Even in the samples that failed at the centre, there was a huge range of variability in the MOE within samples taken from the same pellicle. For example, in dry BC samples from the static-culture, the MOE varied from 25 to 167 MPa. Whereas, the

dry BC samples from a rotating-bioreactor produced at pH 4 varied between 2 to 24 MPa. The BC produced without pH control in the rotating-bioreactor varied between 10 and 72 MPa. This variability could be due to the choice of instrument and the method of conducting the tests or the nature of the samples or a combination of both. Similar variability was observed despite changing the load cell from 50 to 25 N. The average MOE of dry samples produced in the static-culture was 83 ± 52 MPa and the dry samples produced in rotating-bioreactor at pH 4 had an average MOE of 9.7 ± 8 MPa. But the samples produced in the rotating-bioreactor without pH control (no pH control) had a greater tensile strength 35 ± 25 MPa (Fig. 5.14). There was no significant difference between the MOE of dry BC samples produced in a rotating-bioreactor (pH 4 and no pH control) and samples produced in the static-culture ($p > 0.05$ at C.I=95%).

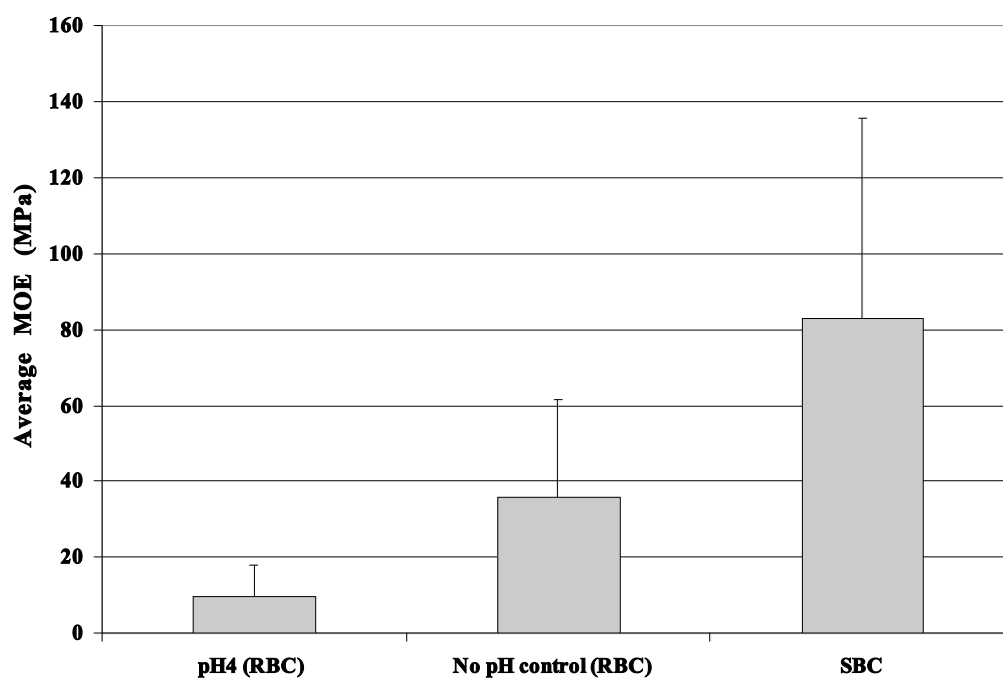


Fig. 5.14 - The average modulus of elasticity (MOE) of dry BC produced in the static-culture and at pH 4 and without pH control in the rotating-bioreactor. The error bars represent the variation between the samples from the same pellicle and different runs (sample size = 15 @ 5 each from 3 different runs).

There was no significant difference in the tensile strength of dry BC produced in the rotating-bioreactor at different tangential velocities and pH because of the huge variability in the tensile strength of the samples tested. The tests were conducted because of the ease of performing tensile tests on dry BC and as a base for comparison with the mechanical tests conducted on wet samples.

5.13.1 Discussion

A tensile strength or MOE of 755 MPa of the dry BC produced in the static-culture was reported by Clasen et al. (2006) while Klemm et al. (2005) reported it between 200-300 MPa. The tensile strength of dry BC produced in the static-culture was reported as 43.68 MPa by George et al. (2005). They also established that there was a reduction in the strength when the wet BC had been boiled in 0.2 M NaOH prior to drying. All the samples used in the dry and wet tests in our study were also boiled in 0.5 M NaOH (Materials and methods, section 2.14) prior to the tests. The mechanical strength could have been compromised to some extent due to the caustic treatment.

The tensile strength of the samples reported in our study could have been enhanced by 10-20% heat pressing the samples (Iguchi et al. 2000) before the mechanical tests, instead oven-drying the samples at 104 °C. They reported the MOE of air-dried BC produced by static-culture as 16,900 MPa while the heat pressed samples ranged between 15,000 to 18,000 MPa. The tensile strength of dry BC samples produced in static-culture were reported by Kornmann et al. (2003) and Krystynowicz et al. (2000) as approximately 30.6 MPa, while that reported by Phisalaphong et al. (2007) was 5.21 MPa. Thus the tensile strength reported by different studies range from 5 to 755 MPa. This difference could be due to the choice of instrument, method of testing and/or the nature of the BC sheets produced by the individual groups or a combination of any of the three probable causes.

A study conducted by Yamanaka (2000) reported that the MOE was impacted by the width of the ribbon because of their uniplaner orientation. This perhaps explains the reason for higher MOE of the BC produced in static-culture than that produced in the rotating-bioreactor. Similarly, the MOE of the BC produced in the rotating-bioreactor without pH control is higher than when the pH was controlled at 4. This can be attributed to the ribbon size as established in Chapter 3, section 3.7.2.1.

There have been limited published reports of BC produced in the rotating-bioreactor, however one such study reported the tensile strength of dry BC produced in the rotating-bioreactor as 22.9 MPa (Krystynowicz et al. 2002). This result is comparable to the tensile strength of the BC produced without pH control in the rotating-bioreactor at 35 ± 25 MPa (Fig. 5.14). This result was used for comparison because their study did not mention active pH control. The huge amount of variability in the samples tested suggests that there could be some issues with the method and/or instrument used for measurement. There could also be differences in the fibrillar orientation of the BC pellicles produced by either method.

5.14 Summary

The WHC is one of the most important properties of BC followed by the mechanical strength. In the present study it was found that there is a difference in the WHC and the mechanical (compressive) strength of the BC produced in both static-culture and rotating-bioreactor. The average WHC of BC produced in static-culture was $92 \text{ g}_{\text{water}}/\text{g}_{\text{BC}}$ and $115 \text{ g}_{\text{water}}/\text{g}_{\text{BC}}$ when produced in RBC, varying different conditions (pH, glucose concentration and tangential velocity). The WHC of the BC produced in RBC increased with the increase in both tangential velocity (0.063 to 0.18 m/s) and pH (3.0 to 6.0) but did not change when the glucose concentration was varied (8 to 77 g/L). The WHC of the

BC produced in static-culture remained unchanged despite varying pH and glucose concentration. The rewettability of the BC was also investigated and it was found that its reduced with the increase in tension (0.5 to 75 cm). The BC was also found to be hygroscopic in the never-dried form and hydrophobic in dried form.

A compression rig and protocol was developed to measure the compressive modulus of wet BC. It was found that the BC produced in the static-culture was stronger (dried and never-dried form) than the one produced in the rotating-bioreactor under different conditions of pH, tangential velocity and glucose concentration. It was also noted that the qualitative analysis of BC produced in RBC gave results in contrast to the results obtained from the compression rig.

CHAPTER 6

Composites

6.1 Introduction

Composites are engineering materials made from two or more components. The most readily available composite in nature is wood made up of cellulose, hemicellulose and lignin (Hoadley 2000). It is a popular trend to use plant-based cellulose fibres to reinforce composites made from synthetic polymers. In addition to plant-based cellulose, the alternate sources such as bacterial cellulose are also gaining importance in recent times. The BC has impressive properties such as high water holding capacity, good absorption rates, biodegradability and non-allergenic nature (Klemm et al. 2005) making it a very good material for high-end medical applications, thus justifying the high cost of production. There are reports in the literature of the application of BC in areas such as wound dressing (Ciechańska 2004; Czaja et al. 2005; Wan and Millon 2005), artificial blood vessels (Bodin et al. 2007a; Klemm et al. 2001), artificial skin (Czaja et al. 2005; Czaja et al. 2007; Jonas and Farah 1998) scaffold for tissue engineering of cartilage (Svensson et al. 2005), dental implants, vascular grafts and catheter covering dressing (Wan and Millon 2005). BC has many attractive properties but sometimes there is a need to alter one or more properties in order to cater to different applications. For example the mechanical strength of BC is poor in its wet state therefore many research groups are focused on trying to improve the same. This is achieved by modification of the BC during production and/or post-production by different methods explained in detail in the next section.

6.2 Composites of bacterial cellulose

In the past, many researchers have tried to modify BC in order to make a novel composite that has properties different from pure BC. Some researchers have modified the BC by physical and /or chemical treatment post-harvest, while others have modified the growth medium by addition of miscible polymers or chemicals that subsequently modify the BC properties (Chanliaud and Gidley 1999; Ciechańska 2004; Ifuku et al. 2007; Lee et al. 2001; Luo et al. 2008; Ogawa and Tokura 1992; Seifert et al. 2004; Whitney et al. 1999; Yano et al. 2008). There are others researchers who have added material like newspaper during production. This material gets physically entrained in the growing BC to give a composite (Mormino and Bungay 2003; Serafica et al. 2002). Irrespective of the method, the composites produced had different properties compared to pure bacterial cellulose (Table 4).

The polymerization and crystallization are two separate processes that occur during the formation of BC (Benziman et al. 1980). Therefore, after the polymerization of BC, the polymer added previously in the medium can co-crystallize during BC formation, producing a closely blended composite material (Benziman et al. 1980). Many novel composites have been produced using the static-culture method by the addition of polymers/chemicals in the culture medium during production such as pectin (Chanliaud and Gidley 1999), xyloglucan (Whitney et al. 1999), chitosan (Ciechańska 2004), carboxymethyl cellulose and polyvinyl alcohol (Seifert et al. 2004). Some other researchers have added collagen (Luo et al. 2008), silica solution (Yano et al. 2008), polylactic acid (PLA) (Piao et al. 2005) and N-acetylglucosamine (Lee et al. 2001; Ogawa and Tokura 1992) in the medium during BC production.

Other researchers have modified the BC post-production, by treating the dry BC with acids such as phosphoric and sulfamic (Svensson et al. 2005), acrylic acid (Choi et al. 2004) and chemicals such as cellulose acetate butyrate (Gindl and Keckes 2004). Some researchers have impregnated dry BC with different resins (Duchemin 2008; Ifuku et al. 2007; Kramer et al. 2006; Yano et al. 2005) while others have dissolved dry BC and chemically treated the same to form a composite (Phisalaphong et al. 2008b; Wang et al. 2008). While some research groups have treated wet (never-dried) BC with co-polymers such as polyethylene glycol (Seves et al. 2001), tetraethoxysilane (Barud et al. 2008) ammonium hexachloropalladate (Evans et al. 2003), gelatin (Lin et al. 2009; Nakayama et al. 2004), carbon nanotubes (Yoon et al. 2006) and silver nitrate (Maneerung et al. 2008; Maria et al. 2010) another group has blended wet BC along with carboxymethyl cellulose to produce a composite (Yudianti and Indrati 2008). In all the mentioned composites, the BC was produced in a static-culture. There are fewer published studies on the production of composites using the rotating-bioreactor. Serafica et al. (2002) investigated the incorporation of different materials, successfully entraining materials such as tiny glass beads, paper fibres, silica gel, talc etc into the BC during production, using the rotating-bioreactor. A similar study has been published by Mormino and Bungay (2003) in which shredded paper was incorporated into the BC during production in a rotating-bioreactor. There are no other published studies about composite production using a rotating-bioreactor.

Table 4 - Properties of BC or composite produced by modification of BC during production or post-production.

Modification process	Polymer/chemical	Properties of modified BC or composite	Reference
During production	Xyloglucan	Improved stiffness and extensibility.	(Whitney et al. 1999)
During production	Pectin	Improved extensibility but reduced stiffness.	(Chanliaud and Gidley 1999)
During production	Chitosan acetate Chitosan lactate	Improved elasticity and water release value in wet composite.	(Ciechańska 2004)
During production	Carboxymethyl cellulose and Methylcellulose	Improved water holding capacity in wet and dry states.	(Seifert et al. 2004)
During production	Polyvinyl alcohol	Reduced water holding capacity.	(Seifert et al. 2004)
During production	N-acetyl-glucosamine	Higher Young's modulus and greater susceptibility to lysozyme.	(Ogawa and Tokura 1992)
During production	Glucosamine and N-acetyl-glucosamine	Higher production of exopolymers	(Lee et al. 2001)
During production	Collagen	Changed the crystalline structure of BC	(Luo et al. 2008)
During production	Silica solution	Elastic modulus increased	(Yano et al. 2008)
During production	Different types of paper	Improved mechanical strength	(Mormino and Bungay 2003)
During production	Sephadex resin beads, Amberlite IRP-64 resins, aluminium particles, silica gel, newspaper and Cellufine powder	Improved mechanical strength, packing for immobilized enzyme bioreactor	(Serafica et al. 2002)
Post-production	Polyethylene glycol	When dried showed lipophilic instead of	(Seves et al. 2001)

		hydrophilic property.	
Post-production	Epoxy, Acrylic and Phenol-formaldehyde	Optically transparent with lower thermal expansion. Increased tensile strength.	(Yano et al. 2005)
Post-production	Phosphoric and Sulfamic acid	Reduced tensile strength. Supports growth of chondrocytes.	(Svensson et al. 2005)
Post-production	Modified Gelatin	Improved re-hydration ability of dried BC	(Lin et al. 2009)
Post-production	Acrylic acid	Improved mechanical properties	(Choi et al. 2004)
Post-production	Cellulose acetate butyrate	Improved mechanical properties	(Gindl and Keckes 2004)
Post-production	Acrylic resin (tricyclodecane dimethanol dimethacrylate)	Reduced refractive index, reduced crystallinity	(Ifuku et al. 2007)
Post-production	Acrylic acid, Acrylate, and methylacrylate	Improved water absorption and mechanical properties	(Kramer et al. 2006)
Post-production	Dissolved in urea and treated with calcium chloride	Novel nanostructure and improved mechanical properties	(Phisalaphong et al. 2008b)
Post-production	Esterified with benzoyl chloride	Thermotropic, liquid crystalline BC formed	(Wang et al. 2008)
Post-production	Tetraethoxysilane	Improved thermostability and broad emission band under UV excitation	(Barud et al. 2008)
Post-production	Ammonium hexachloropalladate	Ability to catalyze precipitation of metals	(Evans et al. 2003)
Post-production	Gelatin	Improved WHC under pressure and mechanical properties	(Nakayama et al. 2004)
Post-production	Multiwalled carbon nanotubes	Improved electrical conductivity	(Yoon et al. 2006)
Post-production	Silver nitrate	Strong anti-microbial activity	(Maneerung et al. 2008)
Post-production	Silver nitrate	Strong bactericidal activity	(Maria et al. 2010)
Post-	Carboxymethyl cellulose and	Improved mechanical	(Yudianti and

production	glycerol	properties	Indrati 2008)
Post-production	Chitosan, Polyethelene glycol and Gelatin	Improved cell adhesion	(Kim et al. 2010)
Post-production	Polylactic acid	Improved elasticity	(Piao et al. 2005)

6.3 Production of composites *in-situ* during BC production using static-culture

In the current study, the production of modified BC was investigated by the addition of different additives into the medium during the BC production using both the static-culture and the rotating-bioreactor. The static-culture was used in order to ascertain the compatibility of the additive with the growth medium and also its dilution factor. In addition, the modified-BC produced in the static-culture provided a baseline for comparison of the same produced in the rotating-bioreactor.

The different additives used *in-situ* to produce BC composites were gelatin, chitosan and chondroitin sulphate. The choice of the co-polymers was based on reports in literature on the usage of gelatin and chitosan to produce modified BC *in-situ* using static-culture. In the present study the feasibility of producing the modified BC *in-situ* using rotating-bioreactor was investigated. The choice of chondroitin sulphate was based on the reports in literature about the medical application. There are no published reports on the use of chondroitin sulphate with BC hence the novelty of the same was explored in the present study. Among the various additives tested in the static-culture, only chitosan and chondroitin sulphate were used to produce modified-BC in the rotating-bioreactor (Chapter 2, Materials and methods, section 2.6). The different dilutions and the respective yields, rate of production and WHC of the modified-BC produced with different additives were determined in static-culture (Table 5). All the static-cultures were produced in polypropylene wide mouth bottles (volume = 100 ml I.D. = 45 mm) five samples were

produced for each additive concentration. An initial glucose concentration of 50 g/L was used in the medium for all the cultures and the initial pH was 4.5 ± 0.5 . The growth phase for all the cultures was 10 days.

It must be noted dilutions above 1% w/v of chitosan in the glucose medium were lost due to yeast contamination. Dilutions in a range of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% w/v of chondroitin sulphate in DIW were added to the medium but BC production was observed only in 0.5% w/v. Dilutions greater than 3% w/v of gelatin did not support BC production.

Table 5 - Gelatin, chitosan and chondroitin sulphate were added to the medium for modification of the BC in static-culture. The average yield, rate and the WHC for modified BC produced at different dilutions were evaluated, the variables represent the standard deviation within sample size = 5 for each of the dilutions.

BC modified with the co-polymers	Conc, (%w/v)	Average yield (g_{BC}/g_{glu})	Average rate of production (g_{BC}/m².day)	Average WHC (g_{water}/g_{BC})
Gelatin	1	0.10 ± 0.002	7.7 ± 1.6	76 ± 3
	2	0.13 ± 0.003	8.8 ± 1.8	70 ± 5
	3	0.08 ± 0.001	5.3 ± 2.0	81 ± 3
Chitosan	0.2	0.05 ± 0.001	3.7 ± 3.7	42 ± 8
	0.5	0.04 ± 0.001	5.5 ± 2.3	53 ± 5
	1	0.042 ± 0.001	3.2 ± 1.9	58 ± 3
Chondroitin sulphate	0.5	0.04 ± 0.004	6.2 ± 2	49 ± 7
Unmodified BC	0	0.12 ± 0.01	13.5 ± 2.5	93 ± 12

The harvested pellicles of the modified BC produced using the different co-polymers did not look different macroscopically from the unmodified BC cellulose pellicles. The rate of production and the WHC was also similar to the pure BC cellulose except the yield of the modified BC produced by the addition of chitosan and chondroitin sulphate was three times lesser than the yield of unmodified BC (Table 6).

6.4 Comparison between BC produced by the addition of chitosan and chondroitin sulphate produced in the static-culture and the rotating-bioreactor

The BC was produced using gelatin, chitosan and chondroitin sulphate were produced in both a static-bioreactor and a rotating-bioreactor. The BC produced using these additives was not referred as composite because the presence of both chitosan and Chondroitin sulphate in the modified BC produced was not directly confirmed. The BC produced using gelatin as an additive was lost due to contamination when produced in the rotating-bioreactor despite repeated attempts, therefore only the BC produced by the addition of chitosan and chondroitin sulphate was compared to unmodified BC.

6.5 Modification of BC using chondroitin sulphate

Chondroitin sulphate is a glycosaminoglycan (GAG), made up of sulfated residues of repeating units of β -D-glucuronate and β -D-N-acetylgalactosamine (Murata and Yokoyama 1985). Chondroitin sulphate (CS) along with other glycosaminoglycans is the major components of extra-cellular matrices, especially cartilage. It is also known to be present in mammalian cell surfaces (Fransson 1987). The strength of compression in articular cartilage is mainly due to the proteoglycan aggrecan (Kashiwagi et al. 2000). This is contributed by the repulsion of charged groups on the molecular glycosaminoglycan (GAG) side chains (Szafranski 2005). The predominant (GAG) is

chondroitin sulphate. Published reports also indicate the role of chondroitin sulphate in wound healing (Cornelissen et al. 2000;Yokozeki et al. 1997). It is also known to have an anti-inflammatory effect and enhances the capacity to regenerate injured bones (Bali et al. 2001).

Higher levels of chondrocyte growth in BC has been observed as compared to tissue culture plastic and alginates (other choices of scaffold for tissue engineering) at similar levels of in vitro immune response (Svensson et al. 2005). Therefore, in the current study it was decided to use chondroitin sulphate (CS) as an additive in the medium in order to modify the BC as it might enhance its abilities as a scaffold for tissue engineering or some other medical based application. This was a novel attempt as there are no published reports of the use of CS and BC together in any form.

The SEM micrographs showed that the modified BC produced in the static-culture was similar to the unmodified BC produced under similar conditions (Fig. 6.1). The BC modified with chondroitin sulphate showed excessive scarring and had a ribbon width of $89 \pm 11\text{nm}$ which is comparable to the ribbon width of $94 \pm 39\text{ nm}$ of unmodified BC produced under similar conditions in the static-culture. During the production of BC using the rotating-bioreactor, 0.5% w/v of chondroitin sulphate was added to the medium. A submersion level of $C_L = 26\%$ and $C_S = 21\%$ 50 g/L initial glucose concentration, pH 4 and 0.095 m/s tangential velocity. The pellicles harvested from the rotating-bioreactor were macroscopically similar to those produced under similar conditions without using chondroitin sulphate. The BC modified with chondroitin sulphate too was structurally similar to unmodified BC produced in the rotating-bioreactor under similar conditions. The network of the ribbons appeared more open with lesser scarring in BC modified with chondroitin sulphate compared to that produced in unmodified BC (Fig. 6.2). The ribbon

width of modified BC at 65 ± 9 nm was comparable to the ribbon width of unmodified BC that ranged at 76 ± 15 nm. The BC modified with chondroitin sulphate had qualitatively lesser mechanical strength than the unmodified BC produced in both the static-culture and the rotating-bioreactor.

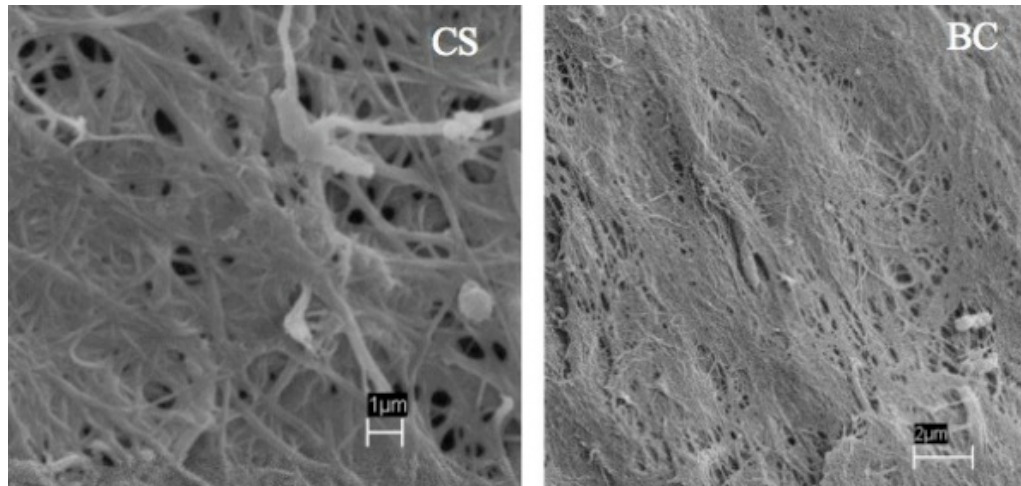


Fig. 6.1 .The SEM micrograph of the BC produced using chondroitin sulphate as an additive (CS) and unmodified BC (BC) produced in a static-culture.

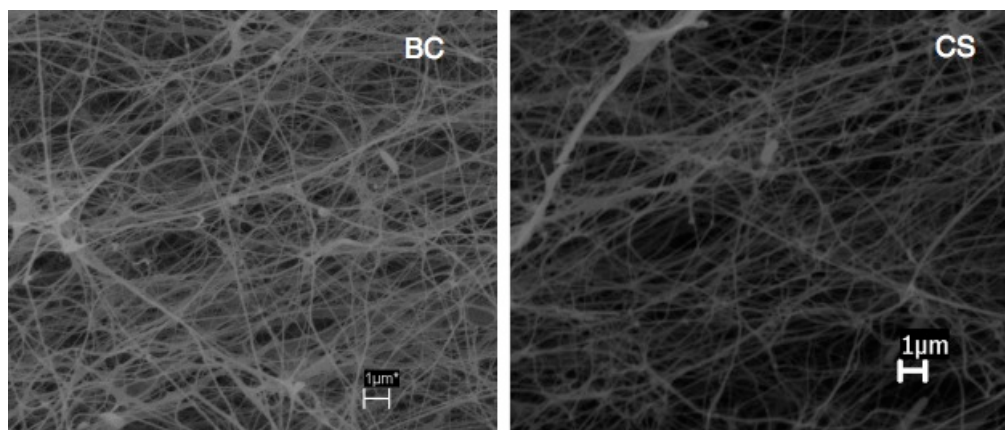


Fig. 6.2 - The SEM micrograph unmodified BC (BC) produced using a rotating-bioreactor and with chondroitin sulphate as an additive (CS).

6.6 Modification of BC using Chitosan

Chitosan (2-acetamido-2-deoxy-b-D-glucopyranose) is a modified carbohydrate derived from chitin (Anthonsen et al. 1993). Chitosan is the fully or partially de-acetylated form of chitin, a linear polysaccharide found in the exoskeletons of crustaceans such as shrimps and crabs and also in the cell walls of some fungi (Bartnicki and Nickerson 1962). It occurs as a random copolymer of GlcNAc and D-glucosamine chitosan, or blocks of sequenced acetylated glucosamines (Aiba 1992). Chitosan is antimicrobial, biocompatible and easily degrades into oligosaccharides that are easily absorbed (Denuziere et al. 1998). Additionally, it can be easily moulded into scaffolds, films and beads (Jarry et al. 2001) making it suitable for tissue engineering. Published studies suggest that chitosan can evoke the differentiation of osteoblast cells and may also enhance the formation of bones (Klokkevold et al. 1996). Previous research has shown that chitosan-based BC composites can be used as wound dressing (Ciechańska 2004). In the present study, modified BC was produced using the static-culture and the rotating-bioreactor, and the physio-mechanical properties of the same, were also investigated.

A submersion level of $C_L = 26\%$ and $C_S = 21\%$, 50 g/L initial glucose concentration, pH 4 and 0.095 m/s tangential velocity was maintained in the rotating-bioreactor. Chitosan, 0.5% w/v was dissolved in 1% v/v of acetic acid was added to the medium. The modified BC produced using chitosan as an additive in the current study was macroscopically similar to the unmodified BC produced under similar conditions. The SEM micrograph of the modified BC produced in the static-culture showed lesser scarring compared to the

unmodified BC and the network of ribbon was distinct with intermittent sheet formation (Fig. 6.3). The ribbon width 91 ± 4 nm of BC modified with chitosan was comparable with the unmodified BC at 94 ± 39 nm (Fig. 6.3).

The SEM micrograph of the modified BC produced in the rotating-bioreactor using chitosan was structurally similar to the unmodified BC produced under similar conditions (Fig. 6.4). The ribbon width of BC modified with chitosan was 69 ± 7 nm and was comparable to the ribbon width of 76 ± 15 nm for unmodified BC produced in rotating-bioreactor under similar conditions.

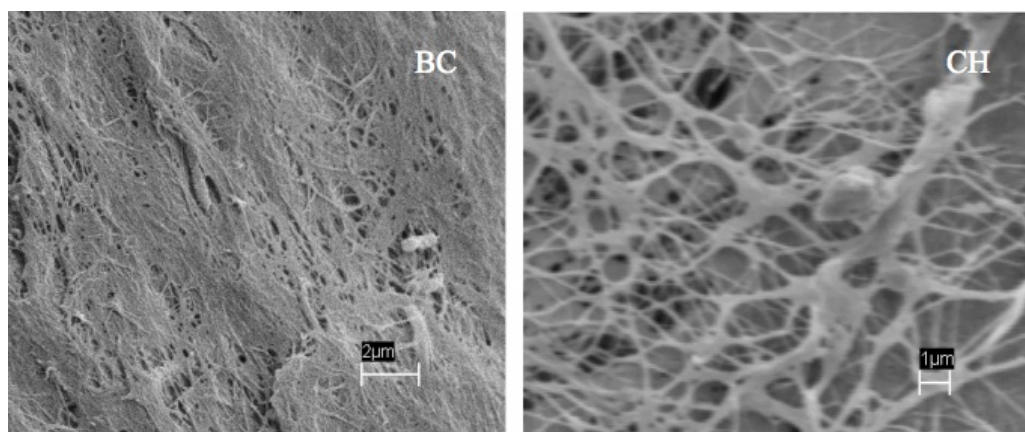


Fig. 6.3 - The SEM micrograph unmodified BC (BC) produced using a static-bioreactor and with chitosan as an additive (CH).

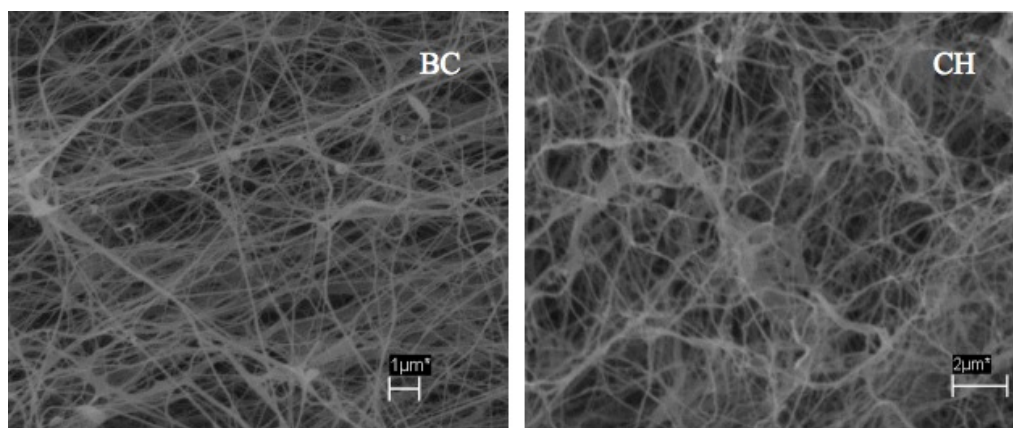


Fig. 6.4 - The SEM micrograph unmodified BC (BC) produced using a rotating-bioreactor and with chitosan as an additive (CH).

6.6.1 Discussion

Morphologically the BC modified with chondroitin sulphate was similar to the unmodified BC when produced in both the static-culture and the rotating-bioreactor under similar conditions (Fig. 6.1 and 6.2). Similarly, the BC modified with chitosan too was structurally similar to the unmodified BC produced in the rotating-bioreactor (Fig. 6.4). The BC modified with chitosan showed a loosely woven network when produced in the static-culture as compared to the unmodified BC (Fig.6.3).

There was no significant difference in the rate of production, yield and WHC of the modified BC when both chondroitin sulphate and chitosan were added to the medium (Table 6). However, a significant difference was noticed in the compressive modulus or the MOE of the modified BC as compared to the unmodified BC. The compressive modulus of the unmodified BC was ten times lesser when BC was modified with chondroitin sulphate and chitosan (Table 5). These results indicate that the addition chondroitin sulphate and chitosan influenced the ribbon formation process of the BC. These results support the the hypothesis that viscosity of the medium changed by addition

of water soluble polymers influence the movement of the bacterial cells affecting the formation of cellulose microfibrils (Shibazaki et al. 1998). Investigative studies should be conducted using techniques such as nuclear magnetic resonance (NMR) and X-ray diffractometry to determine the changes that may have occurred at the molecular level. These studies will also give an estimate of the amount of co-polymer that is incorporated into the unmodified BC and shed light on the crystallinity index and crystal size of the modified BC.

In a similar study where chitosan was used to modify BC produced in static-culture, it was found that glucosamine and N-acetylglucosamine units were incorporated into chains of cellulose (Ciechańska 2004). The degree of polymerization and distribution of molecular weight was determined by conducting gel permeation chromatography (GPC) and structural analysis was performed by Fourier transformed infrared spectrometry (FTIR). The exact method of evaluating the mechanical strength of the wet modified BC was not explained but the results demonstrated that the BC modified with chitosan was stronger (breaking stress=1.02 MPa) compared to the unmodified BC (breaking stress=0.22 MPa). The techniques mentioned in Ciechańska's study were not performed in the current study except the mechanical tests but that in the compression mode. Another difference was that both modified and unmodified BC was produced in static-cultures and not a rotating-bioreactor as in the present study. The results in the present study do not support the results of Ciechańska. It was found that the mechanical strength of the BC was unaffected after modification within the uncertainty of the measurement (Table 5). The mechanical strength was lesser in the present study due to the method of production. It was established (Chapter 5, section 5.11) that the strength of the BC is greatly reduced when produced in a rotating-bioreactor in comparison with BC produced in static-culture.

Table 6 - Comparison of the bioprocessing parameters of unmodified BC with BC modified by the addition of chondroitin sulphate and chitosan in the medium using the rotating-bioreactor. The average yield, rate and the WHC for modified BC produced was estimated the variable represents the standard deviation within the samples with sample size = 10 for each.

Co-polymer	Average rate of production (g_{BC}/m².day)	Yield (g_{BC}/g_{glu})	Average WHC (g_{water}/g_{BC})	Average Compressive modulus (MPa)
Modified with Chondroitin sulphate	2.7± 0.37	0.06 ± 0.001	136 ± 13.5	0.0029 ± 0.0001
Modified with Chitosan	2.7 ± 0.18	0.16 ± 0.002	113 ± 11.44	0.0036 ± 0.00012
Unmodified BC	3.41 ± 0.52	0.17 ± 0.001	108 ± 11.2	0.033 ± 0.00011

The study of composites using both chitosan and chondroitin sulphate was a medical application based study. While the bioactivity assays for the modified BC were not conducted, some preliminary investigations on unmodified BC, produced in the rotating-bioreactor were performed. The Biological assays were performed by Aditya Sharma under the supervision of Dr. George Dias (The University of Otago, Dunedin, New

Zealand). The hen's egg test-chorioallantoic membrane or the HET-CAM test which is used to determine the degree to which a substance can irritate human tissue was performed on BC produced in the rotating-bioreactor produced in the current study. The results were compared to Surgicel®, a commonly used hemostatic agent made up of oxidized cellulose polymer. It was confirmed that BC produced in the rotating-bioreactor was bio-compatible *in-vivo* and non-toxic (Sharma 2009). The cell proliferation assays were not conducted and should be a subject of further study.

6.7. Summary

Modified BC can be produced in-situ in both the static-culture and the rotating-bioreactor using miscible co-polymers. The amount of the co-polymers (chitosan and chondroitin sulphate) present in modified BC was not estimated in the present study. It can be estimated by conducting tests such as NMR, FTIR or GPC.

Chapter 7

Conclusion

There have been a couple of studies in the past investigating the production of BC using the static-cultures and rotating-bioreactors individually, but there are fewer studies involved in the comparative analysis of the same. The present study not only used static-cultures to produced BC in order to establish baseline for the efficiency of the strain in use but also made a comparative analysis with the BC produced in a rotating-bioreactor in varying conditions of growth medium (initial glucose concentration, pH and tangential velocity). Along with the bioprocessing parameters the physio-mechanical properties too were investigated and compared. An attempt was also made to produce BC composites *in-situ* by incorporating miscible co-polymers in the growth medium during production.

The BC produced by the two different methods, static-culture and rotating-bioreactor, were different both macroscopically and microscopically. The BC produced in the static culture was a dense, leathery material characterized by the presence of thicker BC ribbons that showed excessive scarring under SEM and macro-layers in the wet (never-dried) state. In contrast, the BC produced in the rotating-bioreactor was translucent and jelly-like in texture and was made up of fine BC ribbons and that showed lesser scarring under SEM. The reasons for the morphological differences in the BC produced by these two methods were not established but it was speculated that there could be interference in the BC crystallization process due to agitation of the medium in the rotating-bioreactor. This assumption is supported by the X-ray diffraction results in which the crystallinity index of BC produced in the rotating-bioreactor was 83.0% as opposed to that produced in static-culture at 89.4%. Another reason could be due the presence of greater number of cells per unit area in the BC produced in the static-culture compared to that produced in the

rotating-bioreactor. A CSLM protocol was developed using rhodamine blue to stain viable cells was reported for the first time in this study.

No morphological changes were observed within the BC produced in the static-culture at different initial glucose concentrations and pH values. Most of the morphological changes were observed in the BC produced in the rotating-bioreactor at different pH values. The thickness of the BC ribbons reduced with the increase in pH (3-6) as observed in freeze-dried samples (SEM). However, the most interesting difference was observed when the pH was not controlled in the rotating-bioreactor. The BC started resembling the one produced in the static-culture characterized by macro-layers. No studies in the past have reported this observation in BC produced in the rotating-bioreactor. Another interesting observation was made when the pH of the medium was changed from 3 to 4 and vice-versa during the course of the fermentation run. There was not only a change in the compactness of the BC network which was less compact at pH 4 compared to 3, but there was also a change in the direction of the orientation of micro-layers from perpendicular at (pH 3) to parallel (pH 4) to the direction of pellicle growth. Again the reason for this morphological change was not confirmed although the current study hints towards some role of the pH. Further investigations are required in future to confirm the same.

The observed morphological differences between the BC produced in the static-culture and the rotating-bioreactor had a direct impact on the properties of the BC produced by both these methods. The properties tested in the current study were the water holding capacity (WHC) and the mechanical strength. There was a difference in both the WHC and mechanical strength of the BC produced in the static-culture and the rotating-bioreactor. The static-culture produced BC having greater mechanical strength but lesser WHC while the rotating-bioreactor produced BC having greater WHC but lesser strength.

No change in the WHC was observed in the BC samples produced in static culture at different initial glucose concentrations and pH (average WHC $\approx 92 \pm 12$ g_{water}/g_{BC}). Whereas, the WHC increased with the increase in pH (3 to 6) from ≈ 113 to 162 g_{water}/g_{BC} and tangential velocity (0.012 to 0.16 m/s) from ≈ 115 to 176 g_{water}/g_{BC} in the rotating-bioreactor. The WHC was also determined for BC samples produced in rotating-bioreactor at different tensions using a hanging water column of varying height (0.5 to 75 cm). Under tensions of 0.5 and 1 cm there was no significant reduction in the WHC after rewetting. There was a drastic drop in WHC when samples subjected to higher tensions (5 to 75 cm) were rewetted. Additionally, it was confirmed that BC samples can be subjected to tension of 1 cm only once to maintain the WHC. On subjecting the BC samples to the same tension (1 cm) twice there is a loss of WHC by 20%. There was no significant increase when dry BC was rewetted confirming the hydrophobic nature of BC in the dry state.

Compared to WHC assays determining the mechanical strength of the wet BC was a daunting task. The slippery and wet nature of the BC made it very difficult to work with. After a lot of discussions with various groups over a period of time, it was decided to use the compression under submersion as a method for determining the mechanical strength of BC in the wet (never-dried) state. A compression rig was custom built for the same. The modulus of elasticity (MOE) referred to in this study as compressive modulus (CM). It was not significantly different for the samples of BC at different initial pH and glucose concentrations in static culture and was calculated as ≈ 0.46 MPa. The CM of BC produced in the rotating-bioreactor varied between 0.03 to 0.076 MPa with the increase in tangential velocity (0.03 to 0.16 m/s). On a further increase in tangential velocity, there was a reduction in the CM. The qualitative analysis (manual pulling) was different compared to the results from the compression tests. For example, the samples produced in

rotating-bioreactor at pH 6 were stronger than those produced at pH 5, but this observation did not match with the results of the compression test. Additionally no pattern was observed in the CM with the varying pH but the highest CM of ≈ 0.08 MPa was observed in BC produced without pH control. Further development will be required in the compression test method or a totally new method needs to be devised to measure the tensile strength of the wet BC, as it would give a clearer picture of the true mechanical strength of the wet BC. Although there was some ambiguity between measured and qualitative results there was no doubt about the fact that the BC produced in a rotating-bioreactor was much weaker than the BC produced in a static-culture. The procedure for testing the tensile strength of dry BC was relatively straightforward. BC produced in static-cultures was strongest at ≈ 83 MPa while the strongest BC produced in the rotating-bioreactor was without pH control at ≈ 35 MPa.

The production efficiency was estimated by calculating the yield (g of BC produced per g of glucose utilized) and the rate of production (g of BC produced per m^2 per day). The average rate of production and yield was much higher in the BC produced in the static-culture as opposed to the rotating-bioreactor. The yield and rate of production was primarily influenced by the surface area, initial glucose concentration and fermentation period or stage of growth. Besides these factors there was another factor that influenced the rate of production independent of growth stage, surface area or medium called the “wall effect”. It was the ability of the growing pellicle to stick to the sides of the walls of the container opposing the sinking of the growing pellicle into the medium. As of now “the wall effect” was an observation and was not be quantified hence its impact on the production of BC was not estimated. For now it provided an explanation to the difference in the rate of production based on visual observation. The rate of production and yield of BC was influenced by many parameters when produced in a rotating-bioreactor. These

included % submersion of the cylinders in the medium, initial glucose concentration and the tangential velocity of the cylinders. While the varying pH did not have much influence on the rate of production, the highest yield , 0.66 g_{BC}/g_{glu} was produced when the pH was not controlled. A stoichiometric analysis of glucose was carried out and the amount of gluconic acid and acetic acid produced was calculated. Although, the biomass production and CO₂ emission was not calculated the carbon distribution in the products was estimated. Some very interesting observations were made. It was found that gluconic acid was preferentially produced with an increase in glucose concentration in both static culture and the rotating-bioreactor. The production of acetic acid was reduced with an increase in the tangential velocity. The most favourable pH for the production of gluconic acid in the rotating-bioreactor is pH 4 and for acetic acid is pH 3.5. These observations give an insight into the parameters that need to be adjusted to avoid loss of substrate to production of acids.

A novel composite was produced in the rotating-bioreactor by the addition of chondroitin sulphate to the growth medium. Although the composite produced was not tested for the presence of the co-polymers, the physio-mechanical properties were tested. The difference in the mechanical property of the composite in comparison to unmodified BC points towards co-crystallization of the co-polymer. Composite were also produced using chitosan and gelatin.

Future work

The current study investigated the production of BC in the static-culture and the rotating-bioreactor along with the influence of varying the growth conditions on the morphology and properties of the BC produced. To ascertain the factors that influenced these changes some assays are suggested to be conducted in future. Additionally, some changes have been suggested in the reactor to improve the efficiency of the fermentor.

One of the most important observations was the morphological changes observed in the BC produced at different pH using the rotating-bioreactor. The observations in the current study and literature review suggest the role of varying pH and/or dissolved oxygen concentration in the medium. While the pH was monitored online, in the present study the concentration of dissolved oxygen was not monitored and was assumed to be saturated at all times (due to continuous supply of air). BC should be produced and analysed at varying concentrations of dissolved oxygen in the medium while keeping the pH constant to rule out the possibility of the influence of dissolved oxygen on the morphology of BC. Additionally, the oxygen uptake rate should also be calculated for added accuracy to determine the amount of oxygen utilized by the bacterial cells in the growing pellicle.

Additional assays should also be conducted to determine the aspect of the bacteria (physiological, biological and/or morphological) influenced by the change in growth conditions that impacts the BC under production. Simple assays determining the number of BC-producing cells and non-producing mutants to more complex ones including determination of the presence and amount of key enzymes required in the formation of the outer envelope of the bacteria and those required for BC synthesis should be conducted. These assays will help in understanding whether the varying growth conditions affects the bacteria that subsequently impacts BC production and network

formation. This can in turn fuel further research to control the identified parameters in order to produce BC pellicle of desired specification.

Although a custom made rig was developed to test the mechanical strength of wet (never dried) there was a huge amount of difficulty and some of the results under compression were in contrast to qualitative analysis. Hence further protocols should be explored to test the wet BC.

A number of fermentation runs were conducted in the present study but many were lost due to contamination. The source of contamination was identified as the pH probe. The pH probe was not sterilized in an autoclave but by 70% ethanol and inserted after the assembly of the bioreactor. An autoclavable pH probe will minimise the incidence of contamination. Fermentation runs at higher pH can be carried out if the incidence of contaminants can be controlled. Additionally, glucose concentration should be monitored online to reduce the risk of contamination while opening the reservoir for procuring samples.

Another suggestion is to minimize the cellulose (slop) that accumulates on the non-cylinder areas of the reactor. Introducing a stirrer at the bottom of the reactor vessel could minimize it to a large extent. Some other method for level control should be devised to do away with the reservoir as it is the main source of slop. Other areas could be coated with a smooth material such as Teflon as it was observed that BC was only produced on rough surfaces. Provision should be made to monitor CO₂ emissions along with dissolved O₂ and in order to estimate an accurate glucose stoichiometry.

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Appendix 1

Patent report based on bacterial cellulose

Novel strains of cellulose producing bacteria

1. Bio Polymer res. Co. Ltd., Japan.

US 6110712, US 5962278

Tsuchida Takayasu (Japan); Tonouchi Naoto (Japan); Seto Akira (Japan); Kojima Yukiko (Japan); Matsuoka Masanobu (Japan); Yoshinaga Fumihiro (Japan).
A novel strain of *A. xylinus* called *nonacetoxidans*, that resists oxidation by acetates and lactates to a large extent, was isolated.

2. CP Kelco US Inc, USA.

US 6429002, US 6329192, US 5871978, US 5821109, US 5144021, US 5079162, WO 98/41592, EP 0228779

Ben-Bassat Arie (US); Bruner Robert (US); Shoemaker Sharon (US); Aloni Yehoshua (IL); Wong Harry (US); Johnson Donald C (US); Neogi Amar N (US).
A mutated species of the strain *A. xylinus* ATCC Nos. 53264, 53263 and 53524 was isolated, that not only produced less gluconic acids in the medium, but also gave a novel reticulated product.

3. Bio Polymer Res Co Ltd., Japan.

US 6818434, US 6140105, US 5962277, EP 1306388

Watanabe Kunihiro (Japan); Takemura Hiroshi (Japan); Tabuchi Mari (Japan); Tahara Naoki (Japan); Toyosaki Hiroshi (Japan); Morinaga Yasushi (Japan); Tsuchida Takayasu (Japan); Yano Hisato (Japan); Yoshinaga Fumihiro (Japan).
Novel cellulose producing bacteria was isolated, that produced BC having weight-average degree of polymerisation in terms of Polystyrene of 1.6×10^4 or above and Bingham polysaccharide as the by-product.

4. Bio Polymer Res Co Ltd., Japan.

EP 0843017

Naritomi Takaaki (Japan); Kouda Tohru (Japan); Naritomi Michi (Japan); Yano Hisato (Japan); Yoshinaga Fumihiro (Japan).
A process of BC production in agitated culture condition wherein, the BC is produced at a high rate by using a better strain, while keeping a check on the residual sugar and maintaining it at a specific concentration.

5. The University of Texas System, USA.

CA 1334178

Malcolm Brown, R. Jr. (USA) and Chyr-Lin, Fong (Taiwan).
Identification of cellulose producing prokaryotic micro-organisms including, *Acetobacter*, *Agrobacterium*, *Rhizobium*, *Pseudomonas* and *Alcaligenes*.

6. Board of Regents, The University of Texas System, USA.

US 4954439

Brown, Jr.; R. Malcolm (USA), Lin; Fong C. (Taiwan).

Strains of *Acetobacter xylinum* NQ5, ATCC 53582 were identified. These novel strains are capable of reversal of direction during cellulose extrusion producing cellulose ribbon having double the width.

Novel method of BC production

7. Bio Polymer res. Co. Ltd., Japan. US 4912049

Farah; Luiz F. X. (Brazil).

A process of BC production in agitated culture condition wherein the rate of production and yield is increased, while the power required for running the agitator is reduced by controlling the internal pressure of the fermentation tank.

8. Bio Fill Produtos Bioteχνologicos S.A., Brazil. EP 0792935

Kouda Tohru (Japan); Naritomi Takaaki (Japan); Yano Hisato (Japan); Yoshinaga Fumihiro (Japan).

BC produced in static culture at controlled temperatures, are used as artificial skin graft.

9. Levy Nelson Luiz Ferreira; Kurokawa Edna Cristina; Podlech Pablo Angel Sanchez, Brazil. WO 2004/050986

Levy Nelson Luiz Ferreira (Brazil); Kurokawa Edna Cristina (Brazil); Podlech Pablo Angel Sanchez (Brazil).

A process for the large scale production of BC in the wet form under static culture condition was established. The product obtained was of high purity and specific physical and chemical properties.

10. Weyerhaeuser Co., USA. US 4863565

Johnson Donald C (USA); Neogi Amar N (USA).

Sustained BC production under agitated culture condition was achieved using different methods and medium combinations.

11. Bio Polymer res. Co. Ltd., Japan. US 6013490

Kouda Tohru (Japan); Nagata Yasuhisa (Japan); Yano Hisato (Japan); Yoshinaga Fumihiro (Japan).

A cultivating apparatus for BC production under agitated culture conditions was developed wherein the oxygen transfer coefficient was maintained between 25 to 50/hr.

12. Ajinomoto KK, Japan. US 6627419, US 6060289

Ishihara Masaru (Japan); Yamanaka Shigeru (Japan).

A cell division inhibitor was added to the culture medium to modify the cellulose microfibrils that improved their Young's modulus. This can be used in the BC production using any culture condition, e.g. (static, agitated etc).

13. Pharmacia Corp., USA. WO 01/05838

Yang Zhi-Fa; Sharma Sanjeev; Mohan Chat; Kobzeef Joseph.
An improved process for production of re-hydratable and re- dispersible BC using co-
agents and sheer activation under agitated culture conditions.

14. Rensselaer Polytechnic Institute, USA.

US 6071727

Bungay; Henry R. (USA); Serafica; Gonzalo C. (USA).

Production of BC using a rotary disk or linear conveyor bioreactor. The BC produced has
very high water holding capacity.

15. Rensselaer Polytechnic Institute, USA.

US 5955326

Bungay, III; Henry R. (USA); Serafica; Gonzalo C. (USA).

Production of microbial cellulose using a rotating disk film bioreactor.

BC used as a novel product

16. Weyerhaeuser Co., USA.

US 4861427, EP 0289993

Johnson Donald C (USA); Neogi Amar N (USA); Leblanc Henry A (USA).

Wet BC produced under static culture conditions is applied to the surface of paper
during the paper manufacturing process to improve its surface quality.

17. Weyerhaeuser Co., USA.

US 5207826

Westland John A (USA); Stephens R Scott (USA); Johnston Jr. William C (USA);
Rosenkrans Harold J (USA).

BC produced in agitated culture conditions was used as binding agent *in situ*.

18. Monsanto Co., USA.

US 5951910

Skaggs C Bryan (USA); Sifferman Thomas R (USA); Swazey John M (USA); Dial
Harold D (USA); Rakitsky Walter G (USA).

The reticulated BC produced under agitated culture conditions was used as a rheological
modifying agent (agents that provide enhanced cling and flow properties to humectants).

19. Monsanto Co., USA.

US 5637197

Watt Walter D (USA); Adams Terry N (USA); Peterson Gary D (USA); Stephens R Scott
(USA); Askew James M (USA).

A process for applying BC, produced under agitated culture conditions as a coating on a
substrate on a continuous basis, was developed.

20. Weyerhaeuser Co., USA.

US 5362713

Westland John A (USA); Penny Glenn S (USA); Lenk Deborah A (USA).

BC produced under agitated culture conditions was incorporated in drilling mud to
improve its rheological properties.

21. Weyerhaeuser Co., USA.

US 5350528, US 5009797

Westland John A (USA); Penny Glenn S (USA); Stephens R Scott (USA); Winslow Alan R (USA).

BC produced under agitated culture conditions was used to improve the rheological properties for hydraulic fracturing fluids.

**22. Space Environmental Technology Company Inc., and Ajinomoto Co Inc., Japan.
US 6423182**

Furunaga Toshikatsu (Japan); Yamanaka Shigeru (Japan).

Dry BC produced in either agitated or static culture conditions was used to coat plain paper to manufacture recording paper capable of printing superior quality images.

**23. Weyerhaeuser Co., USA.
US 4960763**

Stephens R Scott (USA); Westland John A (USA); Neogi Amar N (USA).

Dry BC produced under agitated culture conditions was used to bind to portions of cholesterol present in aqueous solution in mammals when administered orally.

**24. Weyerhaeuser Co., USA.
US 4919753**

Johnson Donald C (USA); Neogi Amar N (USA).

Wet BC produced under agitated conditions was used as a binder to produce non-woven fabric like product.

**25. Goodyear Tyre & Rubber, USA.
US 5290830**

Tung William (USA); Tung Deborah A (USA); Callander Douglas D (USA); Bauer Richard G (USA).

Dry BC produced under agitated conditions was used to produce reinforced elastomeric articles.

**26. Sony Corp., Japan.
US 5274199**

Uryu Masaru (Japan); Kurihara Noboru (Japan).

Wet BC produced under static conditions was used to manufacture acoustic diaphragm.

**27. Weyerhaeuser Co., USA.
US 5011596**

Shaw Douglas R (USA); Stephens R Scott (USA).

Wet BC produced under agitated condition was used as a depressant for readily floatable silicate minerals in the ore floatation process.

**28. Trustees of TUFTS College, USA.
WO 2006/042287**

Kaplan David L (USA); Wong Peter Y (USA).

Scaffolds for tissue engineering were coated with BC to support cell growth.

Production of BC composite *in situ*

29. The University of Texas System, (US).

CA 1339913, EP 0346507

Malcolm Brown, R. Jr. (USA).

Carboxyl methyl cellulose was added to the culture medium *in situ* to obtain BC composite having modified physio-chemical properties including high absorbency. This composite was produced in static culture condition.

Modification of BC post-production by addition of co-polymers

30. Sony Corp., Tokyo, Japan.

US 2001034383, US 6410618B2

Uryu Masaru (Japan); Tokura Kunihiro (Japan).

BC produced in static culture conditions was dried, powdered and mixed with biodegradable polymeric material to produce a biodegradable composite.

31. UT-Battelle LLC, Oak Ridge, TN, USA.

US 6986963

Evans Barbara R (USA); O'Neill Hugh M (USA); Jansen Valerie Malyvanh (USA); Woodward Jonathan (USA).

Dry BC sheets produced in static culture conditions were placed in salt solutions metal and dried in a manner that the reduced metal gets incorporated into BC. These sheets are used to construct fuel cells.

32. Akerman, Senterfitt & Eidson, PA, USA.

US 2004/096509

Hutchens Stacy A (USA); Woodward Jonathan (Great Britain); Evans Barbara R (USA); O'Neill Hugh M (USA).

BC produced in static culture conditions was treated with soluble calcium salt solution followed by a phosphate salt. The product is used for dental filling and bone implants.

33. Sony Corp., USA.

US 6274652

Uryu Masaru (Japan); Tokura Kunihiro (Japan).

Dry BC produced in static culture conditions was powdered and mixed with biodegradable polymeric material to produce a composite.

34. XYLOS Corporation, USA.

US 6986963

Evans Barbara R (USA); O'Neill Hugh M (USA); Jansen Valerie Malyvanh (USA); Woodward Jonathan (USA)

BC produced under static culture conditions was dehydrated using organic solvents such as methanol, ethanol, propanol, isopropanol, acetone and their mixtures. The solvent was then removed to obtain BC that can be used as human tissue substitutes and bulking agents for plastic.

35. Agency Ind Science Techn.; Sony Corp.; Ajinomoto KK, Japan.

US 4742164

Iguchi Masatoshi (Japan); Mitsuhashi Shigenobu (Japan); Ichimura Kunihiro (Japan); Nishi Yoshio (Japan); Uryu Masaru (Japan); Yamanaka Shigeru (Japan); Watanabe Kunihiro (Japan).

Dry BC produced in static culture conditions was macerated and mixed with different organic or inorganic materials to form sheets of desired properties.

36. Pharmacia Corp., USA.

US 6241812

Smith Barbara A (Great Britain); Colegrove George T (USA); Rakitsky Walter G (USA).
Wet BC produced in agitated culture conditions was mixed with different cationic agents to produce rheological modifying agents and stabilizing agents.

37. Wan Wan-Kei; Million Leonardo, Canada.

WO 2005/016397

Wan Wan-Kei (Canada); Million Leonardo (Canada).
Wet BC produced under static culture conditions was treated with poly vinyl alcohol post production to produce wound dressing.

38. Politechnika Lodzka; Bielecki Stanislaw; Krystynowicz Alina; Czaja Wojciech, Poland.

WO 2005/003366

Bielecki Stanislaw (Poland); Krystynowicz Alina (Poland); Czaja Wojciech (Poland).
Dry, powdered BC was mixed with poly vinyl alcohol solution to produce immobilised biocatalysts.

39. Xylos Corp., USA.

EP 1438975

Serafica Gonzala (USA); Mormino Richard (USA); Hoon Russel (USA).
Wet BC produced under static conditions was rendered non-pyrogenic by a series of chemical washes followed by homogenising. The fluidity of the homogenised BC was controlled by addition of a polyol. The BC gel formed was used as a wound dressing.

40. Xylos Corp., USA.

EP 1473047

Serafica Gonzala (USA); Mormino Richard (USA); Oster Gerry Ann (USA); Kevin E (USA); Koehler Kevin P (USA).
Wet BC produced under static culture conditions was treated with polyhexamethylene biguanide (PHMB) to produce wound dressing.

41. Xylos Corp., USA. CA 2524184, WO 2005/009276, US 2004/013811, US 2004/0142019

Serafica Gonzala (USA); Mormino Richard (USA); Oster Gerry Ann (USA); Kevin E (USA); Koehler Kevin P (USA).
BC produced under static culture conditions was treated with PHMB (p-hydroxymercuribenzoate) post production to produce a wound dressing.

Modification of BC post production by novel treatment

42. Volpe and Koenig, P.C., USA.

US 2006134758

Levy Nelson L F (Brazil); Kurokawa Edna C (Brazil); Podlech Pablo A S (Brazil).

Novel process to obtain wet BC sheets of high purity and specific physio-chemical properties was developed. The product is used as skin substitute.

43. Bio Polymer Res. Co. Ltd., Japan.

US 2006134758

Levy Nelson L F (Brazil); Kurokawa Edna C (Brazil); Podlech Pablo A S (Brazil).

The properties of BC such as dispersibility, suspensibility and viscosity were improved by post- production treatment.

44. Thailand Polymer Res. Co. Ltd., Thailand.

US 5962676

Tammarate Pramote (Thailand).

A modification process for wet BC produced under static or agitated culture conditions, was developed. The resultant product is highly absorbent with reduced stiffness.

45. Bio Polymer Res. Co. Ltd., Japan.

US 5962676

Tammarate Pramote (Thailand).

Properties such as viscosity of dry BC produced under agitated or static culture conditions were restored to that in the wet state by novel methods.

46. Damien Christopher James; Oster Gerry Ann; Beam Heather Ann, USA.

WO 2005/018435

Damien Christopher James (USA); Oster Gerry Ann (USA); Beam Heather Ann (USA).

Wet BC produced in static culture conditions, was dried in a series of processes including freezing, exchanging water with methanol and methanol with supercritical carbon dioxide and finally the carbon dioxide was removed to obtain a dry form. This product can be used as a tissue substitute in surgery.

47. Weyerhaeuser Co., USA.

WO 91/16445

Gupta Maharaja K (USA); Johnson Donald C (USA).

A treatment for removal of discolouration of BC produced in agitated culture conditions was developed that did not compromise the mechanical properties of BC.

48. Imperial Chemical Industries, PLC, Imperial Chemical House, Millbank, London.

EP 0279506, US 4929550, US 5273891

Byrom David, Cleveland (USA).

BC was produced in agitated culture condition in which the amount of carbon source in the growth medium was controlled to produce BC in tablet form that can be used as a bulking agent.

49. Eastman Chemical Company, Kingsport, Tennessee, USA.

US 5360723

John A.H., Kingsport R.M.; Gardner Gray and Scott R.T.

The molecular weight of BC was lowered by addition of 2-deoxy-D-glucose to the growth medium *in situ* in static culture conditions. BC produced in this manner was used as a coating material.

50. XYLOS Corporation, USA.

US 2004/0161453 A1, US 2005/019380

Serafica Gonzalo; Mormino Richard; Oster Gerry Ann; Lentz Kevin and Koehler Kevin (USA).

BC was produced by static culture under oxygen-limiting conditions to control the water content and the thickness of the BC pellicle. The product was used as wound dressing.

51. XYLOS Corporation, USA.

US 2006/0240084 A1, WO 2006/113796 A2

Serafica Gonzalo, Richard Mink, Russell Hoon and Christopher Damien (USA).

BC produced under static culture condition was soaked in different biologically active agents (medicines) and applied to specific wounds acting as a transdermal delivery method.

52. XYLOS Corporation, USA.

US 6599518

Oster Gerry Ann; Lentz Kevin E; Koehler Kevin; Hoon Russell; Serafica Gonzalo and Mormino Richard (USA).

BC produced under static culture conditions was dehydrated using organic solvents such as methanol, ethanol, propanol, isopropanol, acetone and their mixtures. The solvent was then removed to obtain BC that can be used as a human tissue substitute and a bulking agent for plastics.

53. Weyerhaeuser Company, USA.

US 5114849

Ben-Bassat, Arie (USA); Coddington, Kent D (USA); Johnson, Donald C (USA).

The BC production in agitated culture condition was improved by the addition of an agent comprising a polyacrylamide-containing polymer that enhanced the mixing properties of the culture increasing volumetric productivity and yield.

54. Ajinomoto Co. Inc., Japan.

US 6020293, US 5975095, US 5783573

Ahmed Fahim U (USA); Goldschmidt James E (USA); La Cosse Gerald E (USA).

BC produced under static culture condition was modified either by physical or chemical bonding to an animal cell protein and/or substituting hydrogen atoms of at least parts of hydroxyl groups of the cellulose with a positively or negatively charged organic group. This gel is used as a carrier for mass culture of animal cells or as a medical vulnerable cover.

55. The University of Western Ontario, London, Canada.

US 5558861

Yamanaka; Shigeru (Japan); EtoYuzuru (Japan); Takano; Satoshi (Japan); Watanabe, Kunihiro (Japan); Shibai, Hiroshiro (Japan).

BC produced in agitated cultures were dissolved in dimethylacetamide and lithium chloride followed by incorporation of humectants by solvent exchange. This product is used as wound dressings.

Miscellaneous

56. Kay Chemical Co., USA.

US 5846213

Wan, Wan-Kei (USA).

A method of removal and prevention of BC deposits in an aqueous system was developed.

57. Cetus Corp., USA.

US 5268274

Ben-Bassat Arie (USA); Calhoon Roger D (USA); Fear Anna L (USA); Gelfand Moshe (IL).

The nucleic acid sequences encoding the BC synthase operon derived from *Acetobacter* were decoded.

58. IBM, USA.

US 5382565

Bednorz Johannes G (China); Mannhart Jochen D (China); Mueller Carl A (China).

Proteins capable of binding with BC synthase were identified.

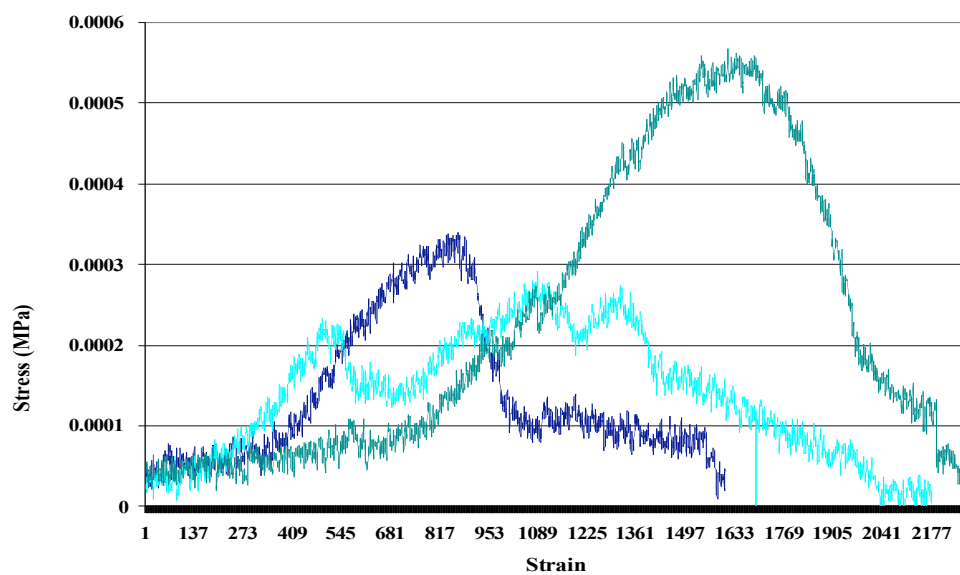
Patent numbers and inventors

US- Patents published by the United States of America

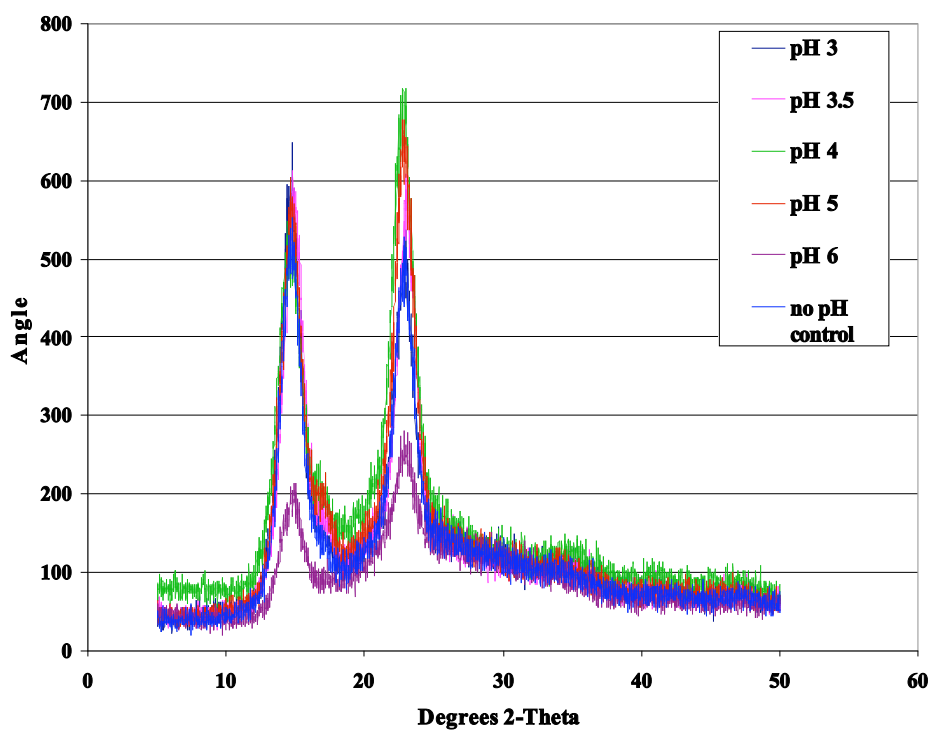
EP –Patents published by European patent office

WO-Patents published by World Intellectual Property

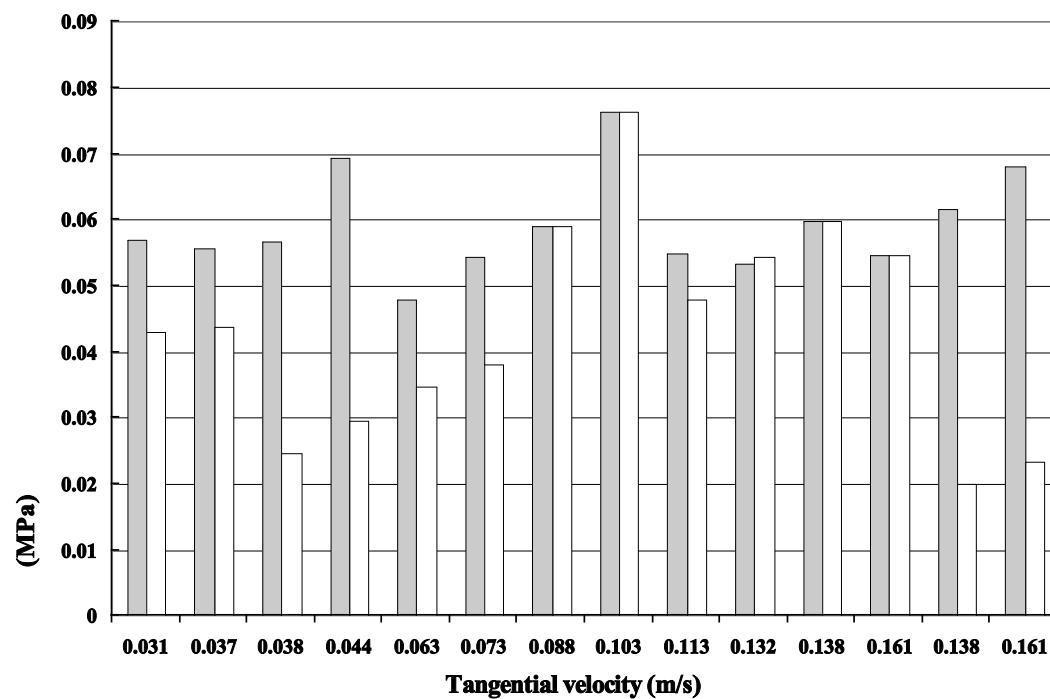
Appendix 2



Tensile tests conducted on wet BC samples produced in rotating-bioreactor using a MTS apparatus.



The X-ray diffraction peak of BC produced at different pH using a rotating-bioreactor



The comparison between the Ultimate compressive stress (white) and the compressive modulus(grey) of BC produced in the rotating-bioreactor at different tangential velocity

Appendix 3

Statistical analysis

1) One-way Analysis of Variance (ANOVA) of the WHC in BC samples produced in the static-culture at different initial glucose concentrations.

The P value is < 0.0001 , considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.897 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
2 g/L vs 11 g/L	59.680	46.861 ***	P<0.001
2 g/L vs 21 g/L	47.820	37.549 ***	P<0.001
2 g/L vs 40 g/L	65.937	51.774 ***	P<0.001
2 g/L vs 46 g/L	54.893	43.103 ***	P<0.001
2 g/L vs 57 g/L	61.663	48.418 ***	P<0.001
2 g/L vs 72 g/L	64.927	50.981 ***	P<0.001
2 g/L vs 87 g/L	44.960	35.303 ***	P<0.001
11 g/L vs 21 g/L	-11.860	9.313 ***	P<0.001
11 g/L vs 40 g/L	6.257	4.913 *	P<0.05
11 g/L vs 46 g/L	-4.787	3.759 ns	P>0.05
11 g/L vs 57 g/L	1.983	1.557 ns	P>0.05
11 g/L vs 72 g/L	5.247	4.120 ns	P>0.05
11 g/L vs 87 g/L	-14.720	11.558 ***	P<0.001
21 g/L vs 40 g/L	18.117	14.225 ***	P<0.001
21 g/L vs 46 g/L	7.073	5.554 *	P<0.05
21 g/L vs 57 g/L	13.843	10.870 ***	P<0.001
21 g/L vs 72 g/L	17.107	13.432 ***	P<0.001
21 g/L vs 87 g/L	-2.860	2.246 ns	P>0.05
40 g/L vs 46 g/L	-11.043	8.671 ***	P<0.001
40 g/L vs 57 g/L	-4.273	3.355 ns	P>0.05
40 g/L vs 72 g/L	-1.010	0.7931 ns	P>0.05
40 g/L vs 87 g/L	-20.977	16.471 ***	P<0.001
46 g/L vs 57 g/L	6.770	5.316 *	P<0.05
46 g/L vs 72 g/L	10.033	7.878 ***	P<0.001
46 g/L vs 87 g/L	-9.933	7.800 ***	P<0.001
57 g/L vs 72 g/L	3.263	2.562 ns	P>0.05
57 g/L vs 87 g/L	-16.703	13.116 ***	P<0.001
72 g/L vs 87 g/L	-19.967	15.678 ***	P<0.001

Mean Difference	95% Confidence Interval Difference	From	To
2 g/L - 11 g/L	59.680	53.443	65.917
2 g/L - 21 g/L	47.820	41.583	54.057
2 g/L - 40 g/L	65.937	59.700	72.173
2 g/L - 46 g/L	54.893	48.657	61.130
2 g/L - 57 g/L	61.663	55.427	67.900
2 g/L - 72 g/L	64.927	58.690	71.163
2 g/L - 87 g/L	44.960	38.723	51.197
11 g/L - 21 g/L	-11.860	-18.097	-5.623
11 g/L - 40 g/L	6.257	0.020	12.493
11 g/L - 46 g/L	-4.787	-11.023	1.450
11 g/L - 57 g/L	1.983	-4.253	8.220
11 g/L - 72 g/L	5.247	-0.989	11.483
11 g/L - 87 g/L	-14.720	-20.957	-8.483
21 g/L - 40 g/L	18.117	11.880	24.353
21 g/L - 46 g/L	7.073	0.836	13.310
21 g/L - 57 g/L	13.843	7.607	20.080
21 g/L - 72 g/L	17.107	10.870	23.343
21 g/L - 87 g/L	-2.860	-9.097	3.377
40 g/L - 46 g/L	-11.043	-17.280	-4.807
40 g/L - 57 g/L	-4.273	-10.510	1.963
40 g/L - 72 g/L	-1.010	-7.247	5.227
40 g/L - 87 g/L	-20.977	-27.213	-14.740
46 g/L - 57 g/L	6.770	0.5334	13.007
46 g/L - 72 g/L	10.033	3.797	16.270
46 g/L - 87 g/L	-9.933	-16.170	-3.697
57 g/L - 72 g/L	3.263	-2.973	9.500
57 g/L - 87 g/L	-16.703	-22.940	-10.467
72 g/L - 87 g/L	-19.967	-26.203	-13.730

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	7	9781.8	1397.4
Residuals (within columns)	16	77.85	4.866
Total	23	9859.7	

$$F = 287.19 = (MStreatment/MSresidual)$$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
2 g/L	5	114.63	0.9097	0.5252	114.78
11 g/L	5	54.947	1.876	1.083	54.780
21 g/L	5	66.807	0.6768	0.3907	67.120
40 g/L	5	48.690	1.426	0.8235	48.760
46 g/L	5	59.733	2.442	1.410	60.500
57 g/L	5	52.963	1.055	0.6088	52.800
72 g/L	5	49.700	2.946	1.701	49.100
87 g/L	5	69.667	4.041	2.333	69.000

Group	95% Confidence Interval			
	Minimum	Maximum	From	To
2 g/L	113.65	115.45	112.37	116.89
11 g/L	53.16	56.900	50.287	59.606
21 g/L	66.03	67.270	65.125	68.488
40 g/L	47.23	50.080	45.147	52.233
46 g/L	57.00	61.700	53.667	65.800
57 g/L	52.00	54.090	50.344	55.583
72 g/L	47.10	52.900	42.381	57.019
87 g/L	66.00	74.000	59.626	79.707

2) One-way Analysis of Variance (ANOVA) of the WHC of BC samples produced in static-culture at different pH (2.4, 4, 5 and 6)

The P value is 0.5289, considered not significant.

Variation among column means is not significantly greater than expected by chance.

Post tests

Post tests were not calculated because the P value was greater than 0.05.

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	3	418.71	139.57
Residuals (within columns)	8	1399.6	174.94
Total	11	1818.3	

$$F = 0.7978 = (MStreatment/MSresidual)$$

Summary of Data

Number of Group	Standard Points	Standard Error of Mean	Standard Deviation	Mean	Median
pH 2.4	5	66.933	9.717	5.610	63.000
pH 4	5	83.217	8.930	5.155	80.340
pH 5	5	74.003	7.221	4.169	75.980
pH 6	5	77.600	21.760	12.563	70.830

Group	95% Confidence Interval			To
	Minimum	Maximum	From	
pH 2.4	59.800	78.000	42.794	91.073
pH 4	76.080	93.230	61.033	105.40
pH 5	66.000	80.030	56.064	91.942
pH 6	60.030	101.94	23.541	131.66

3) One-way Analysis of Variance (ANOVA) of the WHC of BC samples produced in rotating-bioreactor at different tension (0.5,1,5,25,50 and 75 cm)

The P value is < 0.0001 , considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.751 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
0.5 cm vs 1 cm	3.147	0.9591	ns P>0.05
0.5 cm vs 5 cm	5.917	1.803	ns P>0.05
0.5 cm vs 25 cm	152.68	46.540	*** P<0.001
0.5 cm vs 50 cm	153.69	46.845	*** P<0.001
0.5 cm vs 75 cm	153.15	46.683	*** P<0.001
1 cm vs 5 cm	2.770	0.8443	ns P>0.05
1 cm vs 25 cm	149.54	45.580	*** P<0.001
1 cm vs 50 cm	150.54	45.886	*** P<0.001
1 cm vs 75 cm	150.01	45.724	*** P<0.001
5 cm vs 25 cm	146.77	44.736	*** P<0.001
5 cm vs 50 cm	147.77	45.042	*** P<0.001
5 cm vs 75 cm	147.24	44.879	*** P<0.001
25 cm vs 50 cm	1.003	0.3058	ns P>0.05
25 cm vs 75 cm	0.4700	0.1433	ns P>0.05
50 cm vs 75 cm	-0.5333	0.1626	ns P>0.05

Mean Difference	95% Confidence Interval Difference	From	To
0.5 cm - 1 cm	3.147	-12.440	18.733
0.5 cm - 5 cm	5.917	-9.670	21.503
0.5 cm - 25 cm	152.68	137.10	168.27
0.5 cm - 50 cm	153.69	138.10	169.27
0.5 cm - 75 cm	153.15	137.57	168.74
1 cm - 5 cm	2.770	-12.817	18.357
1 cm - 25 cm	149.54	133.95	165.12
1 cm - 50 cm	150.54	134.95	166.13
1 cm - 75 cm	150.01	134.42	165.59
5 cm - 25 cm	146.77	131.18	162.35
5 cm - 50 cm	147.77	132.18	163.36
5 cm - 75 cm	147.24	131.65	162.82
25 cm - 50 cm	1.003	-14.583	16.590
25 cm - 75 cm	0.4700	-15.117	16.057
50 cm - 75 cm	-0.5333	-16.120	15.053

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	101511	20302
Residuals (within columns)	12	387.47	32.289
<hr/>			
Total	17	101899	

$$F = 628.76 = (MS_{\text{treatment}}/MS_{\text{residual}})$$

Summary of Data

Number of Group	Standard Points	Standard Error of Mean	Deviation	Mean	Median
0.5 cm	5	170.98	3.995	2.307	173.02
1 cm	5	167.84	3.931	2.270	166.84
5 cm	5	165.07	10.968	6.333	162.44
25 cm	5	18.300	6.123	3.535	14.850
50 cm	5	17.297	1.198	0.6915	17.870
75 cm	5	17.830	1.756	1.014	17.260

Group	95% Confidence Interval			
	Minimum	Maximum	From	To
0.5 cm	166.38	173.55	161.06	180.91
1 cm	164.50	172.17	158.07	177.60
5 cm	155.65	177.11	137.82	192.32
25 cm	14.680	25.370	3.087	33.513
50 cm	15.920	18.100	14.321	20.272
75 cm	16.430	19.800	13.468	22.192

1) Unpaired t test analysis comparing the average WHC of BC samples produced in rotating-bioreactor drained under tension (0.5,1,5,25 and 75 cm) and the average WHC of the same samples rewetted for 24 hours and drained under similar tension

Unpaired t test

Do the means of Column A and Column B differ significantly?

P value

The two-tailed P value is 0.8677, considered not significant.

$t = 0.1710$ with 10 degrees of freedom.

95% confidence interval

Mean difference = -7.293 (Mean of Column B minus mean of Column A)

The 95% confidence interval of the difference: -102.33 to 87.748

Assumption test: Are the standard deviations equal?

The t test assumes that the columns come from populations with equal SDs.

The following calculations test that assumption.

$$F = 1.191$$

The P value is 0.8524.

This test suggests that the difference between the two SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

The t test assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
Column A	0.3150	0.0635	Yes
Column B	0.3129	0.0675	Yes

Summary of Data

Parameter:	Column A	Column B
Mean:	86.897	79.603
# of points:	6	6
Std deviation:	77.041	70.588
Std error:	31.452	28.817
Minimum:	17.370	15.670
Maximum:	170.98	165.43
Median:	75.195	72.355
Lower 95% CI:	6.034	5.514
Upper 95% CI:	167.76	153.6

1) One-way Analysis of Variance (ANOVA) for rewetting BC samples produced in rotating-bioreactor (draining time = 4 hours, tension= 1cm and cycles =2 rewetting time=12 hours)

The column a is the WHC of samples drained for 4 hours at 1 cm, the column B is the WHC of the samples after rewetting for 12 hours in the 1st cycle and column C is the WHC of the samples after rewetting for 12 hours for the second time or 2nd cycle

The P value is 0.0330, considered significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.339 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value	
Column A vs Column B	15.720	3.003	ns	P>0.05
Column A vs Column C	26.207	5.007	*	P<0.05
Column B vs Column C	10.487	2.003	ns	P>0.05

Mean Difference	95% Confidence Interval Difference		
		From	To
Column A - Column B	15.720	-6.991	38.431
Column A - Column C	26.207	3.496	48.918
Column B - Column C	10.48	-12.224	33.198

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	2	1043.9	521.94
Residuals (within columns)	6	493.14	82.190
Total	8	1537.0	

$$F = 6.350 = (MS_{\text{treatment}} / MS_{\text{residual}})$$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
Column A	5	160.69	5.766	3.329	158.79
Column B	5	144.97	11.468	6.621	141.56
Column C	5	134.49	9.046	5.222	132.41

Group	95% Confidence Interval		From	To
	Minimum	Maximum		
Column A	156.12	167.17	146.37	175.02
Column B	135.60	157.76	116.48	173.46
Column C	126.66	144.39	112.01	156.96

4) **One-way ANOVA for the rewetting of dry BC samples produced in rotating-bioreactor for different duration of time 24, 48 and 96 hours**

The P value is 0.4717, considered not significant.

Variation among column means is not significantly greater than expected by chance.

Post tests

Post tests were not calculated because the P value was greater than 0.05.

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 1.767

The P value is 0.4133.

Bartlett's test suggests that the differences among the SDs is not significant.

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	2	9.801	4.901
Residuals (within columns)	9	53.930	5.992
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Total	11	63.731	

$$F = 0.8178 = (MS_{\text{treatment}} / MS_{\text{residual}})$$

Summary of Data

Number of		Standard Error of			
Group	Points	Mean	Deviation	Mean	Median
24 hours	10	10.169	2.464	1.232	11.107
48 hours	10	12.240	3.182	1.591	12.666
96 hours	10	11.880	1.334	0.6670	12.171

95% Confidence Interval

Group	Minimum	Maximum	From	To
24 hours	6.543	11.918	6.248	14.089
48 hours	8.465	15.165	7.178	17.303

96 hours 10.090 13.090 9.758 14.003

5) One-way Analysis of Variance (ANOVA) of the WHC of BC samples produced in rotating-bioreactor at different tangential velocities

The P value is < 0.0001 , considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.457 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
0.035 m/s vs 0.04 m/s	8.734	1.363 ns	P>0.05
0.035 m/s vs 0.068 m/s	2.349	0.3665 ns	P>0.05
0.035 m/s vs 0.09 m/s	1.606	0.2507 ns	P>0.05
0.035 m/s vs 0.12 m/s	-13.344	2.082 ns	P>0.05
0.035 m/s vs 0.15 m/s	-48.711	7.600 ***	P<0.001
0.035 m/s vs 0.01 m/s	6.530	1.019 ns	P>0.05
0.035 m/s vs 0.05 m/s	0.1087	0.01696 ns	P>0.05
0.04 m/s vs 0.068 m/s	-6.385	0.9961 ns	P>0.05
0.04 m/s vs 0.09 m/s	-7.127	1.112 ns	P>0.05
0.04 m/s vs 0.12 m/s	-22.078	3.445 ns	P>0.05
0.04 m/s vs 0.15 m/s	-57.445	8.963 ***	P<0.001
0.04 m/s vs 0.01 m/s	-2.203	0.3438 ns	P>0.05
0.04 m/s vs 0.05 m/s	-8.625	1.346 ns	P>0.05
0.068 m/s vs 0.09 m/s	-0.7427	0.1159 ns	P>0.05
0.068 m/s vs 0.12 m/s	-15.694	2.449 ns	P>0.05
0.068 m/s vs 0.15 m/s	-51.060	7.967 ***	P<0.001
0.068 m/s vs 0.01 m/s	4.181	0.6524 ns	P>0.05
0.068 m/s vs 0.05 m/s	-2.240	0.3496 ns	P>0.05
0.09 m/s vs 0.12 m/s	-14.951	2.333 ns	P>0.05
0.09 m/s vs 0.15 m/s	-50.317	7.851 ***	P<0.001
0.09 m/s vs 0.01 m/s	4.924	0.7682 ns	P>0.05
0.09 m/s vs 0.05 m/s	-1.498	0.2337 ns	P>0.05
0.12 m/s vs 0.15 m/s	-35.367	5.518 **	P<0.01
0.12 m/s vs 0.01 m/s	19.875	3.101 ns	P>0.05
0.12 m/s vs 0.05 m/s	13.453	2.099 ns	P>0.05
0.15 m/s vs 0.01 m/s	55.241	8.619 ***	P<0.001
0.15 m/s vs 0.05 m/s	48.820	7.617 ***	P<0.001
0.01 m/s vs 0.05 m/s	-6.422	1.002 ns	P>0.05

Mean Difference	95% Confidence Interval Difference	From	To
0.035 m/s - 0.04 m/s	8.734	-19.832	37.300
0.035 m/s - 0.068 m/s	2.349	-26.217	30.915
0.035 m/s - 0.09 m/s	1.606	-26.959	30.172
0.035 m/s - 0.12 m/s	-13.344	-41.910	15.221
0.035 m/s - 0.15 m/s	-48.711	-77.277	-20.145
0.035 m/s - 0.01 m/s	6.530	-22.036	35.096
0.035 m/s - 0.05 m/s	0.1087	-28.457	28.675
0.04 m/s - 0.068 m/s	-6.385	-34.950	22.181
0.04 m/s - 0.09 m/s	-7.127	-35.693	21.439
0.04 m/s - 0.12 m/s	-22.078	-50.644	6.488
0.04 m/s - 0.15 m/s	-57.445	-86.011	-28.879
0.04 m/s - 0.01 m/s	-2.203	-30.769	26.362
0.04 m/s - 0.05 m/s	-8.625	-37.191	19.941
0.068 m/s - 0.09 m/s	-0.7427	-29.309	27.823
0.068 m/s - 0.12 m/s	-15.694	-44.259	12.872
0.068 m/s - 0.15 m/s	-51.060	-79.626	-22.494
0.068 m/s - 0.01 m/s	4.181	-24.385	32.747
0.068 m/s - 0.05 m/s	-2.240	-30.806	26.325
0.09 m/s - 0.12 m/s	-14.951	-43.517	13.615
0.09 m/s - 0.15 m/s	-50.317	-78.883	-21.752
0.09 m/s - 0.01 m/s	4.924	-23.642	33.490
0.09 m/s - 0.05 m/s	-1.498	-30.064	27.068
0.12 m/s - 0.15 m/s	-35.367	-63.932	-6.801
0.12 m/s - 0.01 m/s	19.875	-8.691	48.441
0.12 m/s - 0.05 m/s	13.453	-15.113	42.019
0.15 m/s - 0.01 m/s	55.241	26.675	83.807
0.15 m/s - 0.05 m/s	48.820	20.254	77.386
0.01 m/s - 0.05 m/s	-6.422	-34.987	22.144

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 27.761

The P value is 0.0002.

Bartlett's test suggests that the differences among the SDs is extremely significant.

Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
0.035 m/s	0.2204	>0.10	Yes
0.04 m/s	0.1797	>0.10	Yes
0.068 m/s	0.3862	0.0009	No
0.09 m/s	0.2586	>0.10	Yes
0.12 m/s	0.1322	>0.10	Yes
0.15 m/s	0.2301	>0.10	Yes
0.01 m/s	0.2304	>0.10	Yes
0.05 m/s	0.2169	>0.10	Yes

At least one column failed the normality test with $P < 0.05$.
Consider using a nonparametric test or transforming the data (i.e. converting to logarithms or reciprocals).

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	7	19597	2799.6
Residuals (within columns)	56	18403	328.62
Total	63	38000	

$F = 8.519 = (MS_{\text{treatment}} / MS_{\text{residual}})$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
0.035 m/s	8	107.59	21.169	7.484	103.24
0.04 m/s	8	98.857	12.959	4.582	99.666
0.068 m/s	8	105.24	14.973	5.294	108.20
0.09 m/s	8	105.98	16.440	5.812	109.11
0.12 m/s	8	120.94	17.818	6.300	120.64
0.15 m/s	8	156.30	32.241	11.399	158.60
0.01 m/s	8	101.06	12.346	4.365	98.978
0.05 m/s	8	107.48	3.014	1.066	108.33

Group	95% Confidence Interval		From	To
	Minimum	Maximum		
0.035 m/s	88.410	150.16	89.890	125.29
0.04 m/s	80.333	122.42	88.021	109.69
0.068 m/s	69.568	118.24	92.722	117.76
0.09 m/s	78.688	122.04	92.238	119.73
0.12 m/s	93.709	144.21	106.04	135.83
0.15 m/s	109.42	219.86	129.34	183.26
0.01 m/s	82.721	115.15	90.738	111.38
0.05 m/s	101.57	110.64	104.96	110.00

6) One-way Analysis of Variance (ANOVA) of the WHC of BC produced in the rotating-bioreactor at different pH

The P value is < 0.0001 , considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.225 then the P value is less than 0.05.

Mean Comparison	Difference	q	P value
pH 3 vs pH 3.5	-13.594	2.127	ns $P>0.05$
pH 3 vs pH 4	5.526	0.8649	ns $P>0.05$
pH 3 vs pH 5	-17.520	2.742	ns $P>0.05$
pH 3 vs No pH control	13.988	2.189	ns $P>0.05$
pH 3 vs pH 6	-41.088	6.430	*** $P<0.001$
pH 3.5 vs pH 4	19.120	2.992	ns $P>0.05$
pH 3.5 vs pH 5	-3.927	0.6146	ns $P>0.05$
pH 3.5 vs No pH control	27.582	4.317	* $P<0.05$
pH 3.5 vs pH 6	-27.494	4.303	* $P<0.05$
pH 4 vs pH 5	-23.047	3.607	ns $P>0.05$
pH 4 vs No pH control	8.462	1.324	ns $P>0.05$
pH 4 vs pH 6	-46.614	7.295	*** $P<0.001$
pH 5 vs No pH control	31.508	4.931	* $P<0.05$
pH 5 vs pH 6	-23.567	3.688	ns $P>0.05$
No pH control vs pH 6	-55.076	8.619	*** $P<0.001$

Mean Difference	95% Confidence Interval Difference	From	To
pH 3 - pH 3.5	-13.594	-40.591	13.404
pH 3 - pH 4	5.526	-21.471	32.524
pH 3 - pH 5	-17.520	-44.518	9.477
pH 3 - No pH control	13.988	-13.009	40.985
pH 3 - pH 6	-41.088	-68.085	-14.090
pH 3.5 - pH 4	19.120	-7.878	46.117
pH 3.5 - pH 5	-3.927	-30.924	23.070
pH 3.5 - No pH control	27.582	0.5842	54.579
pH 3.5 - pH 6	-27.494	-54.491	-0.4967
pH 4 - pH 5	-23.047	-50.044	3.951
pH 4 - No pH control	8.462	-18.536	35.459
pH 4 - pH 6	-46.614	-73.611	-19.617
pH 5 - No pH control	31.508	4.511	58.506
pH 5 - pH 6	-23.567	-50.565	3.430
No pH control - pH 6	-55.076	-82.073	-28.078

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 29.503

The P value is < 0.0001.

Bartlett's test suggests that the differences among the SDs is extremely significant. Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
pH 3	0.1638	>0.10	Yes
pH 3.5	0.2042	>0.10	Yes
pH 4	0.2169	>0.10	Yes
pH 5	0.1881	>0.10	Yes
No pH control	0.2780	0.0688	Yes
pH 6	0.2691	0.0913	Yes

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	15548	3109.6
Residuals (within columns)	42	13719	326.63
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Total	47	29266	

$$F = 9.520 = (MS_{\text{treatment}} / MS_{\text{residual}})$$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
pH 3	10	113.01	8.816	3.117	113.66
pH 3.5	10	126.60	18.326	6.479	132.42
pH 4	10	107.48	3.014	1.066	108.33
pH 5	10	130.53	17.789	6.289	130.22
No pH control	10	99.021	13.496	4.772	91.646
pH 6	10	154.10	32.226	11.394	166.77

Group	95% Confidence Interval			
	Minimum	Maximum	From	To
pH 3	96.320	125.85	105.64	120.38
pH 3.5	98.558	146.64	111.28	141.93
pH 4	101.57	110.64	104.96	110.00
pH 5	106.10	164.68	115.65	145.40
No pH control	87.234	116.67	87.736	110.31
pH 6	102.22	185.10	127.15	181.04

7) One-way Analysis of Variance (ANOVA) of the modulus of elasticity (MOE) or compressive modulus(CM) of the BC produced in the rotating-bioreactor at different tangential velocities.

The P value is < 0.0001, considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.750 then the P value is less than 0.05.

Mean Comparison	Difference	q	P value
0.031 m/s vs 0.38 m/s	-0.0009171	2.209	ns P>0.05
0.031 m/s vs 0.044 m/s	0.0005058	1.171	ns P>0.05
0.031 m/s vs 0.063 m/s	0.0001551	0.3589	ns P>0.05
0.031 m/s vs 0.073 m/s	-0.0005627	1.302	ns P>0.05
0.031 m/s vs 0.088 m/s	-0.0005771	1.269	ns P>0.05
0.031 m/s vs 0.103 m/s	-0.003390	7.846	*** P<0.001
0.031 m/s vs 0.113 m/s	-0.0001346	0.3115	ns P>0.05
0.031 m/s vs 0.132 m/s	0.0006958	1.610	ns P>0.05
0.031 m/s vs 0.138 m/s	0.0002240	0.5183	ns P>0.05
0.031 m/s vs 0.161 m/s	0.001107	2.562	ns P>0.05
0.38 m/s vs 0.044 m/s	0.001423	3.293	ns P>0.05
0.38 m/s vs 0.063 m/s	0.001072	2.481	ns P>0.05
0.38 m/s vs 0.073 m/s	0.0003543	0.8200	ns P>0.05
0.38 m/s vs 0.088 m/s	0.0003399	0.7475	ns P>0.05
0.38 m/s vs 0.103 m/s	-0.002473	5.723	** P<0.01
0.38 m/s vs 0.113 m/s	0.0007825	1.811	ns P>0.05
0.38 m/s vs 0.132 m/s	0.001613	3.733	ns P>0.05
0.38 m/s vs 0.138 m/s	0.001141	2.641	ns P>0.05
0.38 m/s vs 0.161 m/s	0.002024	4.684	ns P>0.05
0.044 m/s vs 0.063 m/s	-0.0003508	0.7823	ns P>0.05
0.044 m/s vs 0.073 m/s	-0.001069	2.383	ns P>0.05
0.044 m/s vs 0.088 m/s	-0.001083	2.303	ns P>0.05
0.044 m/s vs 0.103 m/s	-0.003896	8.688	*** P<0.001
0.044 m/s vs 0.113 m/s	-0.0006405	1.428	ns P>0.05
0.044 m/s vs 0.132 m/s	0.0001899	0.4236	ns P>0.05
0.044 m/s vs 0.138 m/s	-0.0002819	0.6287	ns P>0.05
0.044 m/s vs 0.161 m/s	0.0006010	1.340	ns P>0.05
0.063 m/s vs 0.073 m/s	-0.0007178	1.601	ns P>0.05
0.063 m/s vs 0.088 m/s	-0.0007322	1.557	ns P>0.05
0.063 m/s vs 0.103 m/s	-0.003545	7.906	*** P<0.001
0.063 m/s vs 0.113 m/s	-0.0002897	0.6460	ns P>0.05
0.063 m/s vs 0.132 m/s	0.0005407	1.206	ns P>0.05
0.063 m/s vs 0.138 m/s	6.88918333e-	50.1536	ns P>0.05
0.063 m/s vs 0.161 m/s	0.0009518	2.123	ns P>0.05
0.073 m/s vs 0.088 m/s	-1.4385867e-	50.03059	ns P>0.05
0.073 m/s vs 0.103 m/s	-0.002827	6.305	** P<0.01
0.073 m/s vs 0.113 m/s	0.0004281	0.9548	ns P>0.05
0.073 m/s vs 0.132 m/s	0.001259	2.807	ns P>0.05
0.073 m/s vs 0.138 m/s	0.0007867	1.754	ns P>0.05
0.073 m/s vs 0.161 m/s	0.001670	3.723	ns P>0.05
0.088 m/s vs 0.103 m/s	-0.002813	5.981	** P<0.01
0.088 m/s vs 0.113 m/s	0.0004425	0.9409	ns P>0.05
0.088 m/s vs 0.132 m/s	0.001273	2.707	ns P>0.05
0.088 m/s vs 0.138 m/s	0.0008011	1.703	ns P>0.05
0.088 m/s vs 0.161 m/s	0.001684	3.581	ns P>0.05

0.103 m/s vs 0.113 m/s	0.003255	7.260	*** P<0.001
0.103 m/s vs 0.132 m/s	0.004086	9.112	*** P<0.001
0.103 m/s vs 0.138 m/s	0.003614	8.060	*** P<0.001
0.103 m/s vs 0.161 m/s	0.004497	10.029	*** P<0.001
0.113 m/s vs 0.132 m/s	0.0008304	1.852	ns P>0.05
0.113 m/s vs 0.138 m/s	0.0003586	0.7996	ns P>0.05
0.113 m/s vs 0.161 m/s	0.001241	2.769	ns P>0.05
0.132 m/s vs 0.138 m/s	-0.0004718	1.052	ns P>0.05
0.132 m/s vs 0.161 m/s	0.0004111	0.9168	ns P>0.05
0.138 m/s vs 0.161 m/s	0.0008829	1.969	ns P>0.05

Mean Difference	95% Confidence Interval Difference	From	To
0.031 m/s - 0.38 m/s	-0.0009171	-0.002889	0.001055
0.031 m/s - 0.044 m/s	0.0005058	-0.001547	0.002558
0.031 m/s - 0.063 m/s	0.0001551	-0.001898	0.002208
0.031 m/s - 0.073 m/s	-0.0005627	-0.002615	0.001490
0.031 m/s - 0.088 m/s	-0.0005771	-0.002737	0.001583
0.031 m/s - 0.103 m/s	-0.003390	-0.005443	-0.001337
0.031 m/s - 0.113 m/s	-0.0001346	-0.002187	0.001918
0.031 m/s - 0.132 m/s	0.0006958	-0.001357	0.002748
0.031 m/s - 0.138 m/s	0.0002240	-0.001829	0.002277
0.031 m/s - 0.161 m/s	0.001107	-0.0009457	0.003160
0.38 m/s - 0.044 m/s	0.001423	-0.0006297	0.003476
0.38 m/s - 0.063 m/s	0.001072	-0.0009805	0.003125
0.38 m/s - 0.073 m/s	0.0003543	-0.001698	0.002407
0.38 m/s - 0.088 m/s	0.0003399	-0.001820	0.002500
0.38 m/s - 0.103 m/s	-0.002473	-0.004526	-0.0004204
0.38 m/s - 0.113 m/s	0.0007825	-0.001270	0.002835
0.38 m/s - 0.132 m/s	0.001613	-0.0004398	0.003665
0.38 m/s - 0.138 m/s	0.001141	-0.0009116	0.003194
0.38 m/s - 0.161 m/s	0.002024	-2.8673931e	-50.004077
0.044 m/s - 0.063 m/s	-0.0003508	-0.002481	0.001779
0.044 m/s - 0.073 m/s	-0.001069	-0.003199	0.001062
0.044 m/s - 0.088 m/s	-0.001083	-0.003317	0.001151
0.044 m/s - 0.103 m/s	-0.003896	-0.006026	-0.001766
0.044 m/s - 0.113 m/s	-0.0006405	-0.002771	0.001490
0.044 m/s - 0.132 m/s	0.0001899	-0.001940	0.002320
0.044 m/s - 0.138 m/s	-0.0002819	-0.002412	0.001848
0.044 m/s - 0.161 m/s	0.0006010	-0.001529	0.002731
0.063 m/s - 0.073 m/s	-0.0007178	-0.002848	0.001412
0.063 m/s - 0.088 m/s	-0.0007322	-0.002966	0.001502
0.063 m/s - 0.103 m/s	-0.003545	-0.005675	-0.001415
0.063 m/s - 0.113 m/s	-0.0002897	-0.002420	0.001840
0.063 m/s - 0.132 m/s	0.0005407	-0.001589	0.002671
0.063 m/s - 0.138 m/s	6.88918333e-5	-0.002061	0.002199
0.063 m/s - 0.161 m/s	0.0009518	-0.001178	0.003082
0.073 m/s - 0.088 m/s	-1.4385867e-5	-0.002248	0.002220
0.073 m/s - 0.103 m/s	-0.002827	-0.004957	-0.0006972

0.073 m/s - 0.113 m/s	0.0004281	-0.001702	0.002558
0.073 m/s - 0.132 m/s	0.001259	-0.0008716	0.003389
0.073 m/s - 0.138 m/s	0.0007867	-0.001343	0.002917
0.073 m/s - 0.161 m/s	0.001670	-0.0004605	0.003800
0.088 m/s - 0.103 m/s	-0.002813	-0.005047	-0.0005789
0.088 m/s - 0.113 m/s	0.0004425	-0.001792	0.002677
0.088 m/s - 0.132 m/s	0.001273	-0.0009612	0.003507
0.088 m/s - 0.138 m/s	0.0008011	-0.001433	0.003035
0.088 m/s - 0.161 m/s	0.001684	-0.0005501	0.003918
0.103 m/s - 0.113 m/s	0.003255	0.001125	0.005386
0.103 m/s - 0.132 m/s	0.004086	0.001956	0.006216
0.103 m/s - 0.138 m/s	0.003614	0.001484	0.005744
0.103 m/s - 0.161 m/s	0.004497	0.002367	0.006627
0.113 m/s - 0.132 m/s	0.0008304	-0.001300	0.002960
0.113 m/s - 0.138 m/s	0.0003586	-0.001772	0.002489
0.113 m/s - 0.161 m/s	0.001241	-0.0008886	0.003372
0.132 m/s - 0.138 m/s	-0.0004718	-0.002602	0.001658
0.132 m/s - 0.161 m/s	0.0004111	-0.001719	0.002541
0.138 m/s - 0.161 m/s	0.0008829	-0.001247	0.003013

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 51.170

The P value is < 0.0001.

Bartlett's test suggests that the differences among the SDs is extremely significant. Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
0.031 m/s	0.2013	>0.10	Yes
0.38 m/s	0.2633	>0.10	Yes
0.044 m/s	0.2024	>0.10	Yes
0.063 m/s	0.2247	>0.10	Yes
0.073 m/s	0.3295	0.0407	No
0.088 m/s	0.3394	0.0609	Yes
0.103 m/s	0.2899	>0.10	Yes
0.113 m/s	0.2752	>0.10	Yes
0.132 m/s	0.2408	>0.10	Yes

0.138 m/s	0.2547	>0.10	Yes
0.161 m/s	0.1499	>0.10	Yes

At least one column failed the normality test with $P < 0.05$.
Consider using a nonparametric test or transforming the data
(i.e. converting to logarithms or reciprocals).

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	10	8.607434	58.607
Residuals (within columns)	56	6.75589	-51.2064096
Total	66	0.0001536	

$$F = 7.135 = (MS_{\text{treatment}} / MS_{\text{residual}})$$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
0.031 m/s	10	0.002287	0.0006746	0.0002550	0.002397
0.38 m/s	10	0.003204	0.0007122	0.0002692	0.002930
0.044 m/s	10	0.001781	0.0005906	0.0002411	0.001898
0.063 m/s	10	0.002132	0.0005003	0.0002043	0.002259
0.073 m/s	10	0.002849	0.0004968	0.0002028	0.002592
0.088 m/s	10	0.002864	0.001315	0.0005881	0.002588
0.103 m/s	10	0.005677	0.002833	0.001157	0.004273
0.113 m/s	10	0.002421	0.0005061	0.0002066	0.002376
0.132 m/s	10	0.001591	0.0001533	6.259534	0.001623
0.138 m/s	10	0.002063	0.001217	0.0004970	0.001656
0.161 m/s	10	0.001180	0.0005838	0.0002383	0.001103

Group	95% Confidence Interval			
	Minimum	Maximum	From	To
0.031 m/s	0.001511	0.003125	0.001663	0.002911
0.38 m/s	0.002688	0.004720	0.002545	0.003862
0.044 m/s	0.0008314	0.002448	0.001161	0.002401
0.063 m/s	0.001405	0.002650	0.001606	0.002657
0.073 m/s	0.002438	0.003546	0.002328	0.003371
0.088 m/s	0.001770	0.005114	0.001231	0.004496
0.103 m/s	0.003466	0.009819	0.002703	0.008650
0.113 m/s	0.001860	0.003076	0.001890	0.002952
0.132 m/s	0.001368	0.001774	0.001430	0.001752

0.138 m/s	0.0005903	0.003643	0.0007850	0.003340
0.161 m/s	0.0005329	0.002046	0.0005670	0.001793

8) One-way Analysis of Variance (ANOVA) of the compressive modulus(CM) or modulus of elasticity(MOE) of the BC samples produced at different pH in the rotating-bioreactor.

The P value is < 0.0001, considered extremely significant.

Variation among column means is significantly greater than expected by chance.

Tukey-Kramer Multiple Comparisons Test

If the value of q is greater than 4.253 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
pH 3 vs pH 3.5	-0.02835	7.679	*** P<0.001
pH 3 vs pH 4	-0.05259	13.187	*** P<0.001
pH 3 vs pH 5	-0.04604	12.469	*** P<0.001
pH 3 vs pH 6	0.01235	3.231	ns P>0.05
pH 3 vs No pH control	-0.06519	16.347	*** P<0.001
pH 3.5 vs pH 4	-0.02424	6.077	** P<0.01
pH 3.5 vs pH 5	-0.01768	4.790	* P<0.05
pH 3.5 vs pH 6	0.04070	10.650	*** P<0.001
pH 3.5 vs No pH control	-0.03684	9.237	*** P<0.001
pH 4 vs pH 5	0.006552	1.643	ns P>0.05
pH 4 vs pH 6	0.06494	15.807	*** P<0.001
pH 4 vs No pH control	-0.01260	2.956	ns P>0.05
pH 5 vs pH 6	0.05839	15.277	*** P<0.001
pH 5 vs No pH control	-0.01915	4.803	* P<0.05
pH 6 vs No pH control	-0.07754	18.874	*** P<0.001

Difference	Mean Difference	95% Confidence Interval	
		From	To
pH 3 - pH 3.5	-0.02835	-0.04406	-0.01265
pH 3 - pH 4	-0.05259	-0.06955	-0.03563
pH 3 - pH 5	-0.04604	-0.06174	-0.03034
pH 3 - pH 6	0.01235	-0.003905	0.02860
pH 3 - No pH control	-0.06519	-0.08215	-0.04823
pH 3.5 - pH 4	-0.02424	-0.04120	-0.007275
pH 3.5 - pH 5	-0.01768	-0.03339	-0.001981
pH 3.5 - pH 6	0.04070	0.02445	0.05696
pH 3.5 - No pH control	-0.03684	-0.05380	-0.01988

pH 4 - pH 5	0.006552	-0.01041	0.02351
pH 4 - pH 6	0.06494	0.04747	0.08241
pH 4 - No pH control	-0.01260	-0.03074	0.005530
pH 5 - pH 6	0.05839	0.04213	0.07464
pH 5 - No pH control	-0.01915	-0.03612	-0.002193
pH 6 - No pH control	-0.07754	-0.09502	-0.06007

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 46.833

The P value is < 0.0001.

Bartlett's test suggests that the differences among the SDs is extremely significant.

Since ANOVA assumes populations with equal SDs, you should consider transforming your data (reciprocal or log) or selecting a nonparametric test.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
pH 3	0.1630	>0.10	Yes
pH 3.5	0.2256	>0.10	Yes
pH 4	0.1934	>0.10	Yes
pH 5	0.1837	>0.10	Yes
pH 6	0.3312	0.0196	No
No pH control	0.2937	>0.10	Yes

At least one column failed the normality test with $P < 0.05$.

Consider using a nonparametric test or transforming the data (i.e. converting to logarithms or reciprocals).

Intermediate calculations. ANOVA table

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	5	0.03220	0.006440
Residuals (within columns)	37	0.004035	0.0001091

Total 42 0.03623

$F = 59.044 = (MS_{\text{treatment}}/MS_{\text{residual}})$

Summary of Data

Group	Number of Points	Standard Mean	Standard Error of Deviation	Mean	Median
pH 3	10	0.01534	0.003385	0.001197	0.01538
pH 3.5	10	0.04369	0.009984	0.003530	0.04083
pH 4	10	0.06793	0.006202	0.002532	0.06812
pH 5	10	0.06137	0.02071	0.007320	0.06635
pH 6	10	0.002987	0.001151	0.0004352	0.002643
No pH control	10	0.08053	0.003353	0.001369	0.08076

Group	95% Confidence Interval			
	Minimum	Maximum	From	To
pH 3	0.009871	0.02060	0.01251	0.01817
pH 3.5	0.03365	0.06038	0.03534	0.05204
pH 4	0.06093	0.07674	0.06142	0.07444
pH 5	0.03563	0.08695	0.04406	0.07869
pH 6	0.002177	0.005518	0.001922	0.004052
No pH control	0.07636	0.08366	0.07701	0.08405

Appendix 4

Calculation of:

Yield = $\frac{g_{\text{dry cellulose}}}{g_{\text{glucose}}}$ expressed as $\frac{g_{BC}}{g_{glu}}$

$$Y_p = \frac{(m_{\text{dry cellulose}})}{[(c_i \times V_i) - (c_f \times V_f)]}$$

$m_{\text{cellulose}}$: $(m_{\text{dry pellicle 1}} + m_{\text{dry pellicle 2}}) [g_{\text{dry cellulose}}]$

$m_{\text{dry waste cellulose}}$: mass of dry cellulose lost as waste $[g_{\text{dry cellulose}}]$

c_i : glucose concentration at the beginning of exponential growth $[g_{\text{glucose}}/L]$

c_f : glucose concentration at harvest $[g_{\text{glucose}}/L]$

V_i : Volume of medium at the start of exponential growth/at inoculation (L)

V_f : Free volume of medium at harvest (L) + medium within cellulose

medium within cellulose = the medium present in both the cellulose which can be calculated using WHC.

Rate of production = $\frac{g_{\text{dry cellulose}}}{m^2 \cdot d}$ expressed as $\frac{g_{BC}}{m^2 \cdot \text{day}}$

$$R_p = \frac{(m_{\text{dry cellulose}})}{[(C_b + C_s)(\text{day})]}$$

Surface area of cylinder = (circumference)(length) = πDL

Surface area of big cylinder (C_b) = $\pi (140 \text{ cm}) (120 \text{ mm}) = 52,752 \text{ mm}^2$

Surface area of small cylinder (C_s) = $\pi (120 \text{ cm}) (120 \text{ mm}) = 45,216 \text{ mm}^2$

$C_b + C_s = 97968 \text{ mm}^2$

$g_{\text{dry cellulose}} = m_{\text{dry cellulose}}$

$m_{\text{dry cellulose}} = (m_{\text{dry pellicle 1}} + m_{\text{dry pellicle 2}}) g_{\text{dry cellulose}}$

day = The number of days of cellulose growth from the date of inoculation till the date of harvest in case of static-culture and the day when the drop in pH was observed till day of harvest in rotating-bioreactor.

Water Holding Capacity = $\frac{g_{\text{water}}}{g_{BC}}$

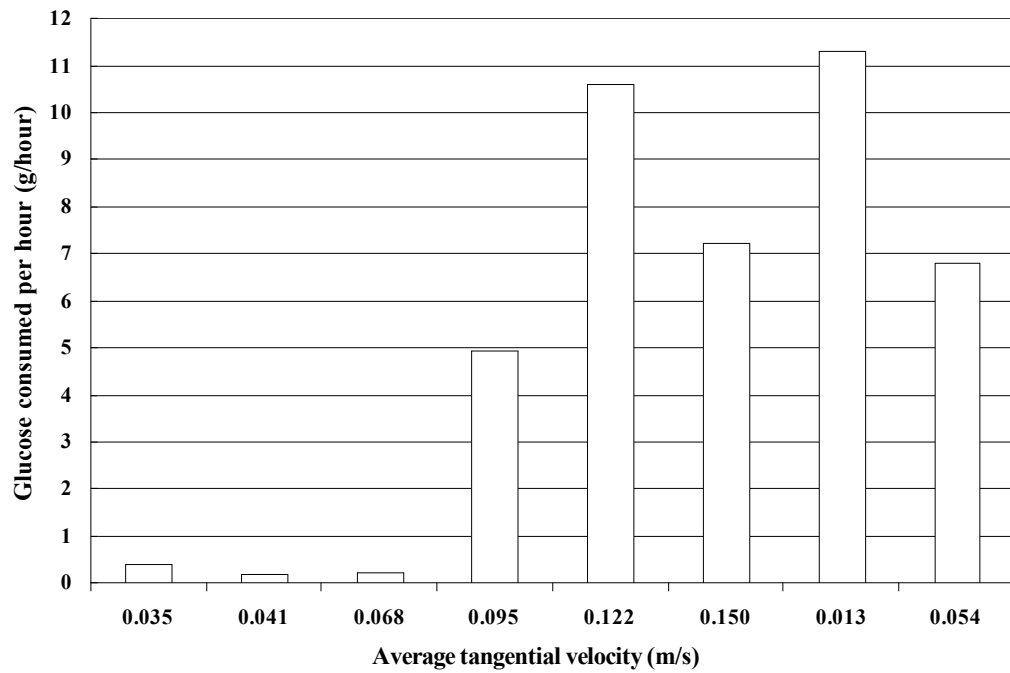
$$WHC = \frac{(g_{\text{wet cellulose}} - g_{\text{dry cellulose}})}{g_{\text{dry cellulose}}}$$

$g_{\text{wet cellulose}}$ - the wet weight of samples after preconditioning at 1 cm tension

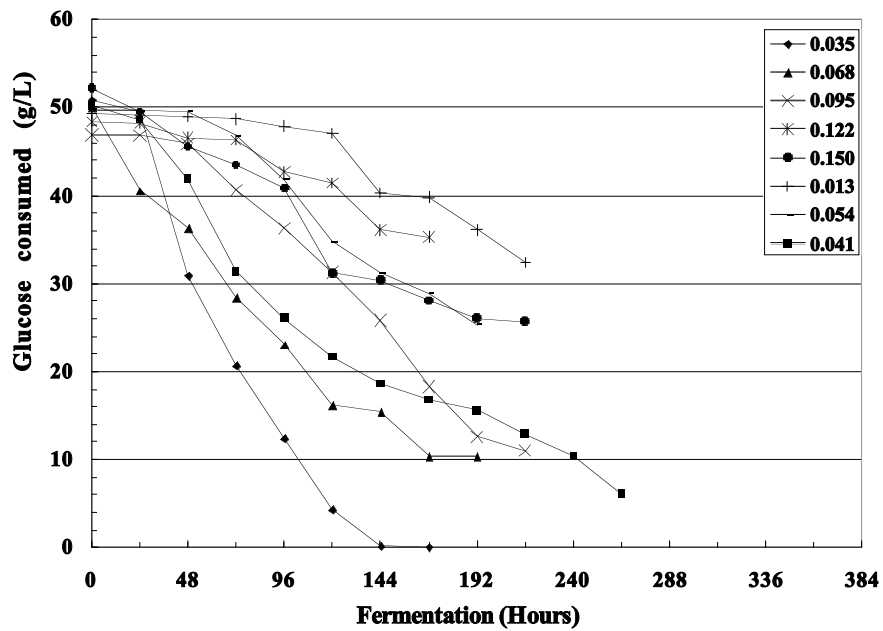
$g_{\text{dry cellulose}}$ - dry weight of the sample

Sample diameter = 25 mm area = 1964 mm^2 .

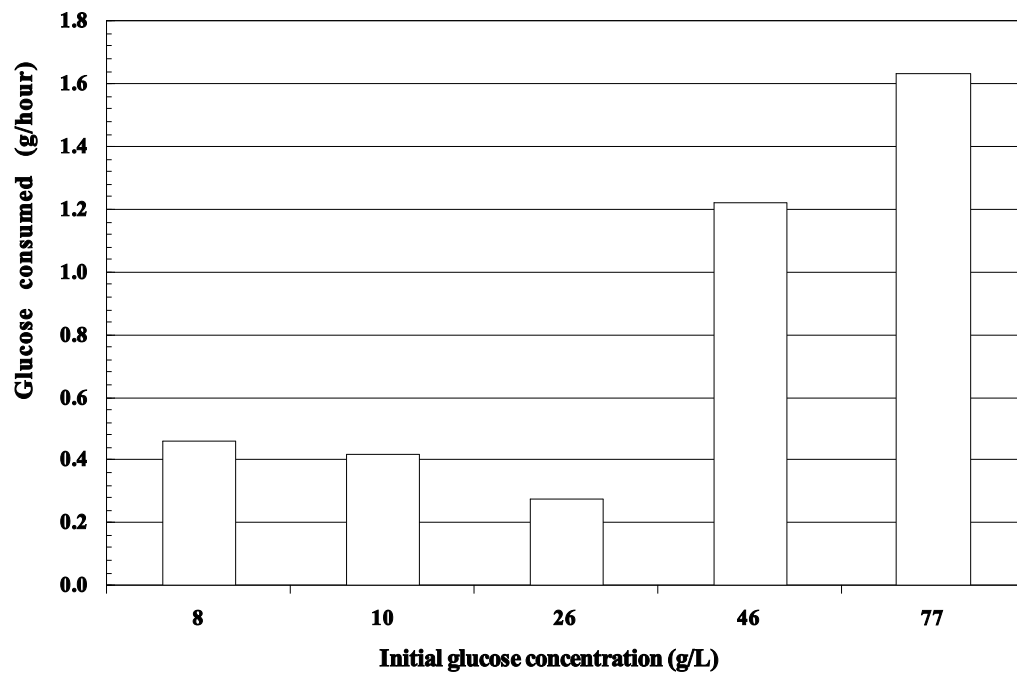
Appendix 5



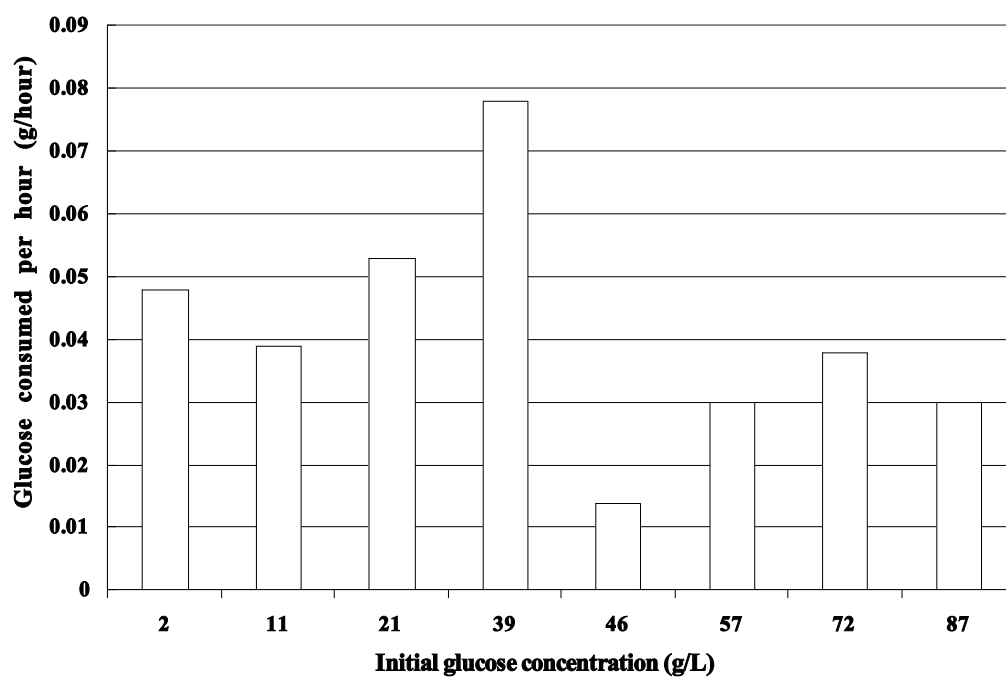
Amount of glucose utilized per hour during the BC production using a rotating-bioreactor at different tangential velocities



Total glucose consumed during BC production using a rotating-bioreactor different tangential velocities.



Amount of glucose utilized per hour during the BC production using a rotating-bioreactor at initial concentration of glucose.



Amount of glucose utilized during BC production using a static-culture at different initial glucose concentration.